Determination of Serum Di-(2-ethylhexyl) Phthalate and Bisphenol A Level in Children with Idiopathic Central Precocious Puberty

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Purpose : Environmental endocrine disruptors may affect the endocrine system or sexual development in children. Several recent studies have focused on indoor and dietary pollutants such as di-(2-ethylhexyl) phthalate (DEHP), which is used as a plastic softener, and bisphenol A, which is used in food-packaging materials. Despite the accumulation of data and the substantial arguments implicatinf these pollutants in endocrinal disorders, no clear evidence has been established yet. Thus, we assessed the serum levels of DEHP and bisphenol A in patients diagnosed with idiopathic central precocious puberty (CPP) to estimate the possible environmental hazards associated with increasing number of cases of precocious puberty.

Methods: The study included 30 patients (29 girls and 1 boy) with idiopathic CPP and 30 normal control children who visited the pediatric endocrinology clinic. CPP was diagnosed on the basis of clinical & hormonal tests, including the gonadotropin-releasing hormone-stimulation test. The serum DEHP and bisphenol A levels were analyzed by gas chromatography/mass spectrometry.

Results : The mean chronological ages (CA) in the idiopathic CPP and control groups were similar (8.6±0.9 vs. 7.8±1.1 years). However, the overall growth stage in the CPP group was advanced. The CPP group had a significantly higher height SDS (1.3±1.0 vs. -0.4±1.1, P<0.005) and weight SDS (1.3±1.3 vs. -0.2±1.3, P<0.005) than the control group. Bone age was significantly more advanced in the CPP group than in the control group (BA-CA : 14.6±9.4 months vs. 0.8±15.3 months, P<0.005). While the serum bisphenol A levels were not significantly different between the groups (11.2±10.3 vs.16.2±12.5 ng/mL), the DEHP levels were significantly higher in the CPP group than in the control group (159.01±92.78 vs. 103.55±92.98 ng/mL, P<0.05).

Conclusion : This study suggests that DEHP, one of most commonly used plasticizers, may be an etiologic factor for precocious puberty. **(J Korean Soc Pediatr Endocrinol 2009;14:154-162)**

Key Words : Di-(2-ethylhexyl) phthalate, Bisphenol A, Endocrine disruptors, Precocious puberty

Introduction

Puberty is characterized by rapid physiological changes such as growth spurt and maturation of the gonads and the brain. It entails the individual's transition period from a non-reproductive to a reproductive state experiencing emotional stress and vulnerability to socio-environmental factors[1]. Puberty is considered precocious when the onset of puberty begins before the age of 8 years in girls, and before the age of 9 years in boys. Pubertal timing is from the awakening of complex neuroendocrine machinery, by the increase of pulsatile secretion of gonadotropin releasing hormone (GnRH) in the hypothalamic-pututary-gonadal axis regulated by excitatory and inhibitory signals, but the primary mechanism is still unclear[2]. Pubertal timing may be determined by various factors including nutritional, intrauterine conditions, stress, climate condition and light darkness cycle, and certain genetic

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factors such as A2 polymorphism of CYP17 gene[3], Oct-2 gene[4], GPR54/KiSS-1[5, 6] at the neuroendocrine level.

In a recent report, the increasing incidence of advanced pubertal timing has been observed worldwide[7-9]. This secular trend of advance was also apparent in Korean children and adolescents[10]. Moreover, Massart etal.[11] reported high incidence of central precocious puberty in a bounded geographic area of northwest Tuscany, indicating environmental factors are involved as major central precocious puberty (CPP) determinants in this area. These findings suggest that environmental factors may play a role on the timing and progression of puberty.

In epidemiologic studies, the potential role of environmental endocrine disrupting chemicals (EDCs) on pubertal development in the human was provided by studies showing increased level of phthalate esters in the serum of young Puerto Rican girls with premature breast development[12, 13], and study showing high levels of 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene (p,p'-DDE) in 26 immigrant girls with precocious puberty[14]. EDCs are a large group of compounds existing in the environment, defined as exogenous substance or materials that alter the function(s) of the endocrine system and consequently cause adverse health effects in the intact organisms, their progeny or population[15]. Over decades, the environmental chemicals have been suspected to alter the pubertal timing[16-18].

Recently, public attention has been focused on the indoor and dietary pollutants such as bisphenol A (BPA) within the food packaging materials or phthalates used as plasticizer. BPA is widely used in the hard plastic products as polycarbonate, epoxy resins for baby bottles, pacifiers, lining of food, drink cans, toys, dental sealants, computers, cell phones, paints, adhesives, enamels, and some microwavable/reusable food packages[19]. The fact that human exposure to BPA is widespread has been identified from the analysis of human urine samples with the primary route of exposure by food[20]. They are found to cause reproductive toxicities[21]. BPA also had been acknowledged as an estrogenic chemical able to interact with human estrogen receptors (ERs)[22].

DEHP, di-(2-ethylhexyl) phthalate, is ubiquitously distributed, which are used within PVCs products, including plastic toys, floor tiles, wall papers, adhesives or vinyl medical devices. They off-gas and are present in residential indoor air. People are at risk of exposure because the phthalates can be absorbed through the skin, inhaled, ingested, or directly administered to patients through transfusions or other medical procedures that use vinyl medical devices[19]. They accumulate in the environment and their exposure has been reported to cause reproductive toxicity, reproductive organ malformations or cancers in the laboratory animals[23]. Adverse health effects reported in human include alteration of lipid metabolism, hepatic peroxisome proliferation, carcinogenicity, premature menarche, estrous cycle/ovulatory irregularities, decreased semen quality, reduced fertility, fetal loss, endometriosis, and malformations of reproductive tract[24, 25]. However, very limited numbers of reports described the endocrine disrupting effects from exposure to EDCs especially in the children. As far as the children are exposed constantly, and concerning the apparent secular trend of precocious puberty, the effects of these widespread chemicals on children's health need to be clarified.

In this study we hypothesized that increased exposure to DEHP or BPA may disrupt the endocrine feedback loops for normal pubertal development. Thus we assessed the serum levels of DEHP and BPA among the patients diagnosed with idiopathic CPP to estimate the exposure level in association with advanced pubertal development in children.

Materials and Methods

1. Subjects

The study subjects were selected among the children, living in Seoul and Kyoung-gi area, who visited the endocrinology section of the pediatric clinic (Gangnam Severance Hospital, Seoul, Korea) for the evaluation of precocious pubertal development. The study subjects including twenty-nine females and a boy were confirmed with idiopathic CPP. The parallel group for control included thirty healthy children, without any evidence of endocrine disease or pubertal signs, selected within the clinic at the same period. Diagnostic process of idiopathic central precocious puberty is described below.

2. Anthropometry and Radiologic Assessment

Clinical and anthropometric evaluation was performed by a same attending physician. Each subject had pubertal develop-

ment evaluation by the same pediatric endocrinologist according to the method of Marshall-Tanner[26]. Bone age view evaluation at the left wrist and hand was performed with Greulich-Pyle method[27]. Brain MRI (magnetic resonance imaging) was done in each subject after final diagnosis of CPP by blood tests to rule out organic lesion.

3. Blood Sample Analysis

Whole blood samples were collected by venipuncture into glass tubes. Routine chemistry and hormone assay (as below) were performed. Remaining samples were centrifuged and stored at -20℃ immediately. The levels of DEHP and BPA in the sera were detected and analyzed by gas chromatography and mass spectra (GC/MS) method. While dealing with the samples, glass devices were used throughout the procedure.

A. Hormonal Assay: Baseline estradiol (in girls), testosterone (in a boy), the baseline luteinizing hormone (LH) and the follicle stimulating hormone (FSH) levels were analyzed from the blood samples. The diagnosis of CPP was confirmed according to the definition as follows[28]; (A) Pubertal sign appeared before the age of 8 in girls and before 9 in boys, (B) with increased growth rate, advanced bone age. (C) Pubertal levels of stimulated LH (>5 IU/L) and LH/FSH ratio (>1) after intravenous LHRH (LH releasing hormone) stimulation test. The stimulated LH and FSH peak levels were measured by radioimmunoassay after administration of gonadorelin, a synthetic LHRH, 100 $\mu g/m^2$ (Relefact, Handok, Seoul, Korea) intravenously.

B. Gas Chromatography/Mass Spectrometry (GC/MS): The frozen sera of the subjects and the normal children, numbered from P1 to 30 and N1 to 30 respectively, were pretreated and extracted for the analysis of BPA and DEHP levels. A GC/MS was performed on an Agilent 6890 GC (Hewlett-Packard, GA, USA) equipped with a 5973 mass selective detector. An Agilent 6890 GC interfaced with a Pegasus® III time-of-flight mass spectrometry (Leco Corp, St. Joseph, MI, USA) was used for GC/TOF-MS for separation of complex mixtures. An Ultra-2 capillary column of 25 m length, 0.20 mm inner diameter, and 0.11 μm of film thickness was used for separation. For the analysis, the initial oven temperature was set to 150°C with duration of 1 min and a following ramp of 10℃/min until 260℃, targeting 320℃ with duration of 2 minutes. The injector, transfer line, and ion source temperatures were held at 280°C, 280°C, and 230°C, respectively. Extracted sample volume of 2 μL was injected spitlessly on GC inlet. Mass spectra were acquired with a scan range of 50-400 *m/z*. Chromatogram acquisition, data handling, automated peak deconvolution, library search, and retention index calculation were done by the Leco Chroma TOF software (v1.61).

4. Statistical Analysis

Statistical significance was determined using SPSS software version 11.5 (SPSS, Chicago, IL, USA). To determine statistical differences between the clinical features of the two groups, we used paired-sample t-test. Correlations among the levels of chemicals and the clinical features, biomarkers from the laboratory test were estimated with Pearson's correlation analysis method. For all statistical tests, results were considered significant at $P<0.05$. Values in figures are mean±SE.

Results

1. Characteristics of the Subjects

Comparisons of clinical features between the CPP and normal subjects are given in Table 1. The mean chronological ages were 8.6±0.9 years in CPP group versus 7.8±1.1 years in the control. Tanner stage was B3±0.9 vs. B1. None of them experienced menarche. No brain lesion was identified by brain MRI. The overall growth of CPP group showed remarkable advance. The height standard deviation scores (SDS) was

Table 1. Clinical and Auxological Characteristics of the Subjects

	Idiopathic CPP	Normal Control	*P*-Value*
N (F:M)	30 (29 : 1)	30 (29 : 1)	
Age (year)	8.6±0.9	7.8±1.1	NS
Height SDS	1.3±1.0	−0.4±1.1	<0.005
Weight SDS	1.3±1.3	−0.2±1.3	<0.005
BMI (kg/m^2)	17.8±1.4	16.2±1.8	<0.005
BA (year)	9.5±1.1	7.9±1.9	<0.005
BA advance:BA−CA (month)	14.6±9.4	0.8±15.3	<0.005
YGR (cm/yr)	7.4±1.9	4.6±0.9	<0.005

Abbreviations : CPP, central precocious puberty; SDS, standard deviation score; BMI, body mass index; YGR , yearly growth rate; BA, bone age; CA, chronological age, NS: Not significant; F, female; M, male
Values are mean±SE
*Estimated by paired T-test

higher in CPP group compared to control group (1.3±1.0 vs. -0.4±1.1, P<0.005), as well as weight SDS (1.3±1.3 vs. -0.2±1.3, P<0.005).The mean body mass indexes (BMI) were increased in the CPP group (17.6±1.5 kg/m^2 vs. 16.2±1.8 kg/m^2, P=0.003). However the percentiles of BMI of both groups were within the normal distribution. Bone ages were 9.5±1.1 years in CPP group vs. 7.9±1.9 years in control group (P< 0.005). Bone age (BA) was advanced in CPP group compared to control group (BA-CA:14.6±9.4 months vs. 0.8±15.3 months, P<0.005).The mean yearly growth rate (YGR) was higher in CPP group than that in control group (7.4±1.9 cm/yr vs. 4.6±0.9 cm/yr, P<0.005).Birth history was not remarkable between CPP group and control group (mean gestational age, 38±0.1 wks vs. 40±0.2 wks; birth weight, 3.2±0.1 kg vs. 3.1±0.1 kg).

2. Hormone Assay

The baseline LH, FSH, and estradiol levels were increased in children with idiopathic CPP. The levels of peak LH and FSH after GnRH stimulation were also markedly increased in children with CPP (Table 2).

Among the clinical and hormonal features measured from the CPP group, bone ages showed significant correlation with the height SDS (correlation r=0.414, P=0.037), weight SDS (r=0.383, P=0.037), baseline LH level (r=0.424, P=0.019), peak LH (r=0.584, P=0.004) and peak FSH (r=0.714, P=0.000) levels in the serum.

Table 2. Hormonal Characteristics of the Children with Idiopathic Central Precocious Puberty

	Idiopathic CPP
LH basal (IU/L)	0.9±1.5
FSH basal (IU/L)	4.7±8.2
Estradiol basal (girls) (pg/mL)	27.6±24.0
Testosterone basal (boy) (pg/mL)	6.5
Stimulated LH peak (IU/L)	22.9±15
Stimulated FSH peak (IU/L)	11.8±3.9
LH peak /LH basal	81.4±70.6
FSH peak/FSH basal	5.4±4.6
LH peak/FSH peak	2.0±1.1

Abbreviation: LH, luteinizing hormone; FSH, follicle stimulating hormone; CPP, central precocious puberty
Values are mean±SE

3. Screening and Quantitative Analysis of Serum DEHP and BPA

Figure 1 represents the extracted ion chromatogram for DEHP and BPA concentrations. Ion peaks are confirmed with the mass spectra acquired from a scan range of 50~400 *m/z*, and calculated by mean of calibration curves (R2>0.99). The concentrations are measured in the range of nanograms per mililiter. The serum levels of BPA were detected from zero to 35.1 ng/mL in CPP group, and 2.9 ng/mL to 45.6 ng/mL in control. DEHP detection levels ranged from 59.45 ng/mL to 541.71 ng/mL in CPP group and from zero to 386.62 ng/mL in control. Serum BPA levels did not show significant difference between CPP group and control group (11.2±10.3 ng/mL vs. 16.2±12.5 ng/mL). Meanwhile, DEHP levels were significantly higher in CPP group than control group (159.01±92.78 ng/mL vs. 103.55±92.98 ng/mL, P<0.05)(Table3).

4. Correlations between Serum DEHP vs. Bone Age, Height SDS, Serum Estradiol and Peak LH

Next, we investigated the correlation between serum DEHP level and the clinical and hormonal parameters measured from the CPP group. The biomarkers such as bone age, height SDS, peak LH, peak FSH, and basal estradiol level were increased in the patients with CPP. However, no significant correlations were observed between serum DEHP level and other parameters (Fig. 2).

Discussion

Most of the epidemiologic studies reported the effect of EDCs on developmental or reproductive system changes found in the areas of accidental contamination[29]. However the outcome of exposure to the environmentally relevant levels of active chemicals is not clear yet. In this study, we

Table 3. Serum Bisphenol A and DEHP Levels in the Children with Idiopathic Central Precocious Puberty

Average	Idiopathic CPP	Normal control	*P*-Value *
Bisphenol A (ng/mL)	11.2±10.3	16.2±12.5	NS
DEHP (ng/mL)	159.01±92.78	103.55±92.98	<0.05

Abbreviations : DEHP, di-(2-ethylhexyl) phthalate; CPP, central precocious puberty; NS, not significant
Values are mean±SE
*Estimated by paired T-test

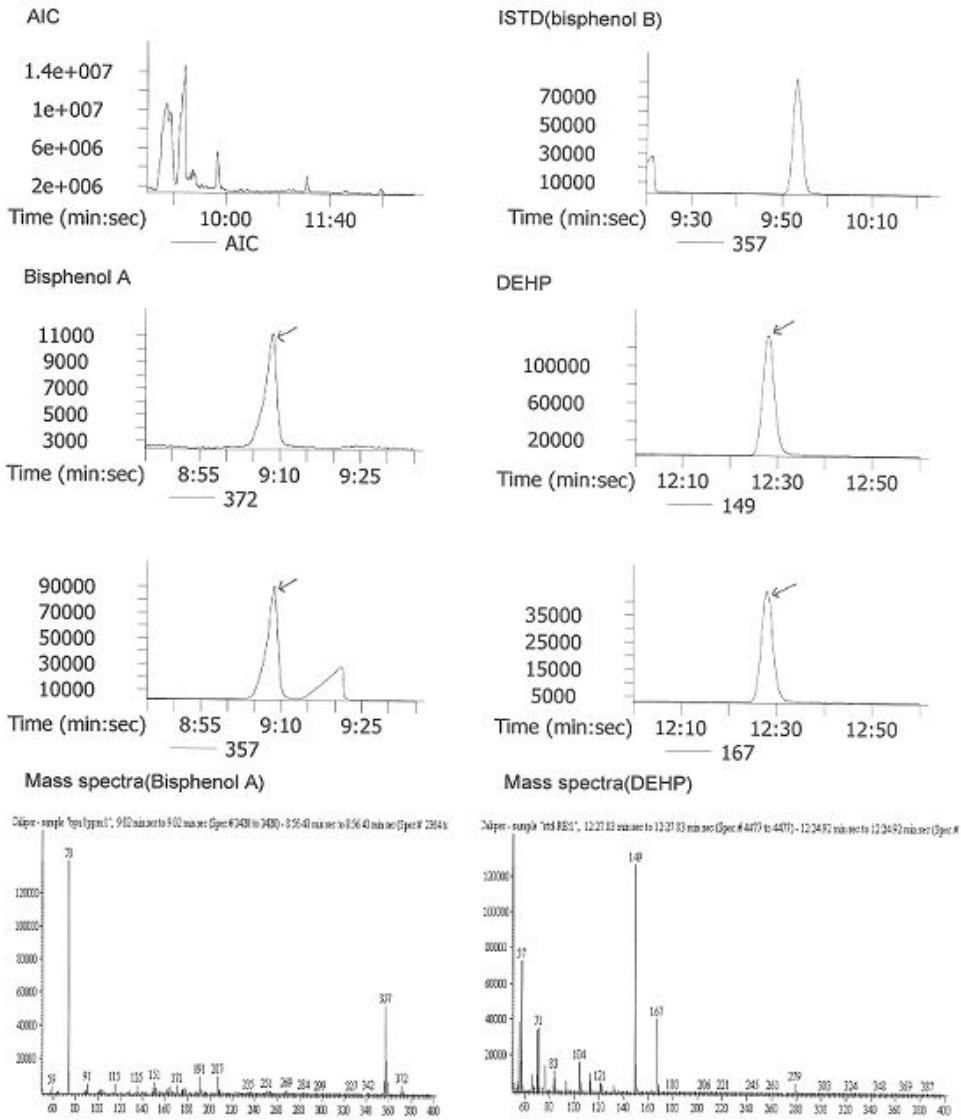

Fig. 1. Representative results of adjusted ion chromatogram (AIC), extracted ion chromatogram and mass spectra of bisphenol A and di-(2-ethylhexyl) phthalate analyzed by gas chromatography/mass spectrometry. Ion peaks are confirmed with the mass spectra acquired from a scan range of 50-400 *m/z* and calculated by mean of calibration curves (R^2>0.99). The concentrations are measured in the range of nanograms per milliliter.

found that the serum levels of DEHP measured with a GC/MS were significantly increased in the children with idiopathic CPP. These relatively high levels of DEHP in patients with CPP seem to be significant, while we have to confirm the reports dealing with high risk of exposure to DEHP in the daily products in Korea. Unfortunately, very few relevant data from epidemiological studies are available to investigate the possible associations between the environmental exposures to DEHP and reproductive health in Korea.

In Puerto Rico, a group of pediatric endocrinologists observed an alarming increase of precocious puberty, and a marked elevation of DEHP in 41 girls experiencing premature thelarche (mean DEHP of 445 ng/mL) as compared to 35 age-matched controls[12, 13]. However, the report was followed by a controversy over the clinical relevance judging from the pharmacokinetic or toxicological standpoint[30]. The influences of phthalates on health have been evaluated through toxicological manner at high doses and mostly in the laboratory animals. In the meantime, certain amount of adverse effects of EDCs results from exposure during fetal development or the non-dose-dependent responses[31, 32]. The investigation between dose-dependent response and clinical outcomes seems to be

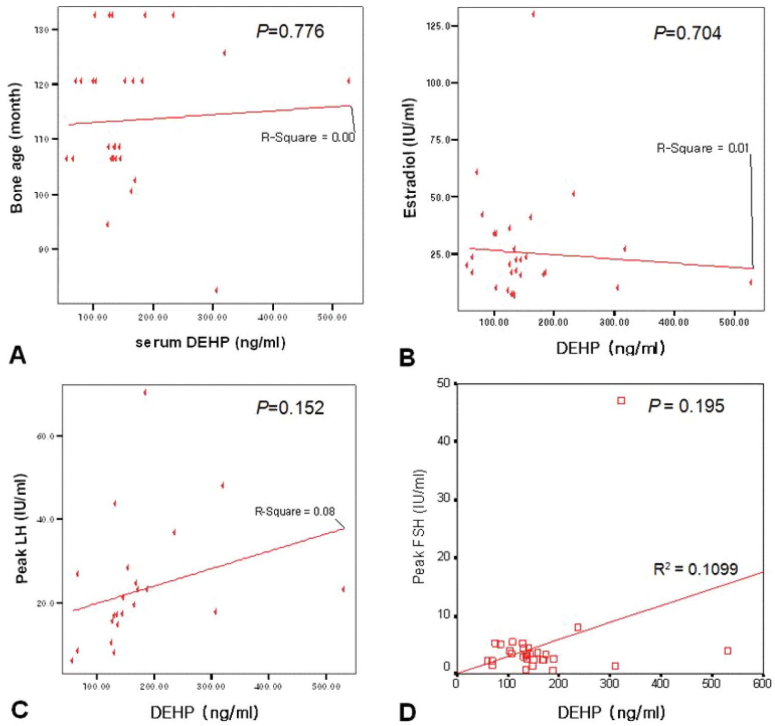

Fig. 2. Serum levels of di-(2-ethylhexyl)phthalate (DEHP) in the children diagnosed with idiopathic central precocious puberty and correlation with clinical and hormonal features. DEHP levels showed no significant association with (A) bone age (month) (B) estradiol (C) peak luteinizing hormone (LH) and (D) peak follicle stimulating hormone (FSH) levels after luteinizing hormone releasing hormone stimulation.

complicated due to non-monotonic, low or high dose-, non-linear responses of phthalates in the body. *In vivo*, at a low level of exposure, phthalates were demonstrated to advance puberty in female mice[33]. Meanwhile, the aromatase activity in granulosa cells was inhibited and testosterone level was reduced in male rats by the phthalate exposure, which is described as anti-estrogenic effect[34].

While the simple quantification of exposure to DEHP in human seems to be not possible yet, blood and urine samples from thousands of Americans have been tested by Center for Disease Control and Prevention (CDC). The results were announced that DEHP levels in blood and urine were low within the safety range[35]. Nonetheless, DEHP is no longer used to manufacture children's products intended for mouthing in the United States, Canada and European countries. Furthermore, Consumer Product Safety Improvement Act of 2008: Sec 108 in the United States recently banned on sale of certain products containing phthalates in children's toys and care articles. Permanent prohibition of DEHP, dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), dibutyl phthalate (DBP), or benzyl butyl phthalate (BBP) and interim prohibition of diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), or di-n-octyl phthalate (DnOP) will be activated sooner or later[36].

Exposures to phthalate esters are widespread and occur at higher levels than previously anticipated. The age at most exposure level was noted to be from 6 month to 4 year of age[37]. Fetuses and younger children are known to be more susceptible due to their rapid growth, mouthing and sucking behaviors. Higher exposure to DEHP in younger children is due to higher food consumption related to their low body weight, behaviors of mouthing, and playing near the ground[38]. Our concern should be focusing on the possible exposure to younger children or fetuses in rapid growing period, the critical window of susceptibility.

We estimated the correlation of the DEHP levels with the clinical and hormonal features in CPP. The serum level of the DEHP didn't show any significant association with clinical and hormonal parameters featuring the pubertal development, such as height SDS, weight SDS, baseline LH, peak LH, and peak FSH levels. Further studies comprising more abundant

numbers of patient will be needed to exploit the role of DEHP on precocious puberty. DEHP has been suggested to act through its metabolite monoethylhexyl phthalate, which suppress the aromatase activity in the ovary, or through a receptor-mediated signaling pathway to alter estradiol production in the ovary[34]. This might refer to the hypotheses that multiple modes of action of EDC may exert effects on transcriptional regulation of target gene expression at the neuroendocrine level. ER-mediated transcription has been shown to be activated by BPA and phthalate through interaction with TRAP220[39]. The research into the mechanism of action of DEHP must be broadened to this area.

Although the BPA levels we measured didn't show difference between the groups, the detection of BPA in the children still remains great concern to us. The European Commission Scientific Committee calculated in 2002 that the daily intake of BPA is much higher in younger children[15]. Several studies have suggested that absorption and distribution of BPA in maternal organs and fetuses are extremely rapid and BPA can easily pass through the placenta after oral administration to pregnant rats[40]. These reflect strong needs to carefully monitor and search for any possible harm of the chemical in the susceptible population in rapid growth.

A potential limitation of our study is that this study was single-centered, the cross-sectional design, without the familial, socioeconomic status or lifestyle adjustment. It should be extended to the larger studies within the general population level with a statistical elaboration. Also, considering the standard detection method, clinical interpretation should be supported by further pharmacokinetic data (e.g. urine levels, metabolites), which are difficult to acquire from the general population especially in children. Lastly, as pubertal timing may affected by various conditions as suggested previously, confounding factors should be adjusted in the future projects. Environmental monitoring data with baseline demographic references are lacking in Korea. Longitudinal studies of full life cycle, including prenatal exposures and multigeneration study would be needed. Data collection through basic researches invitroandinvivoshould leverage to develop surrogate biomarkers of exposure, the causality to adverse effects in human and to understand epigenome.

The levels of serum DEHP were substantially increased in the patients with idiopathic CPP compared to the normal children. The results support a possible effect of DEHP, one of the most commonly used plasticizers, on pubertal development. It may act as one of compound etiologic factors in triggering the puberty. DEHP may act in the various manners within the neuroendocrine system. Further clinical evidences to clarify the mechanism must be done with laboratory data which may be translated to human.

한 글 요 약

특발성 진성 성조숙증 환아에서 측정한 혈장 Di-(2-ethylhexyl) phthalate 및 bisphenol-A 농도

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목 적 : 플라스틱 가소제로 흔히 사용되는 di-(2-ethylhexyl) phthalate (DEHP) 및 각종 식품 포장용기의 소재인 bisphenol A는 에스트로겐 유사 작용이 있다고 알려져 있으며, 소아에서는 성조숙증 발병과의 연관성이 보고된 바 있으나 충분한 연구가 부족한 실정이다. 본 연구는 특발성 진성 성조숙증 환아를 대상으로 내분비교란물질로 알려진 DEHP 및 bisphenol A의 혈중 농도를 측정하여 연관성 여부를 확인하고자 하였다.

방 법 : 외래 방문 환자 중 특발성 진성 성조숙증 환아 30명(여아 29명, 남아 1명)과 정상 대조군 30명(여아 29명, 남아 1명)을 대상으로 하였다. 신체 검진 및 골연령을 측정하였으며, 혈중 성호르몬 농도 및 성선자극호르몬방출호르몬 자극검사를 시행하여 성조숙증을 진단하였다. 혈장 DEHP 및 bisphenol A의 농도는 gas chromatography/mass spectrometry (GC/MS) 방법으로 분석하였다.

결 과 : 성조숙증군과 정상대조군의 평균 연령은 8.6±0.9세 대 7.8±1.1세였으며, 신장 표준편차점수는 1.3±1.0 대 -0.4±1.1(P<0.005),체중 표준편차점수는 1.3±1.3 대 -0.2± 1.3(P<0.005),체질량지수는 17.6±1.5 kg/m^2 대 16.2±1.8 kg/m^2 (P<0.005),골연령은 9.5±1.1세 대 7.9±1.9세(P<0.005)로 성조숙증군의 신체발육이 앞서 있었다. Bisphenol A 검출농도는 두 군간에 차이가 없었으나(성조숙증군 11.2±10.3 ng/mL 대 정상대조군 16.2±12.5 ng/mL) DEHP의 농도는 성조숙증군에서 의미있게 증가한 결과를 보였다(159.01±92.78 ng/mL 대 103.55±92.98 ng/mL, P<0.05).

결 론 : 본 연구에서 특발성 진성 성조숙증 환자에서 증가된 혈중 DEHP의 농도는 성조숙증의 발생 원인 중의 하나로

작용할 가능성을 시사한다. 그러나 채취 과정에서 검체가 오염되는 경우를 배제할 수 없고, 질환 발병과 무관한 인체 노출 범위가 아직까지 확립되지 않았다는 문제가 있다. 향후 내분비교란물질과 성조숙증 발생 사이의 연관성에 대한 보다 광범위한 연구와 내분비교란물질의 동물 및 인체 내 작용 및 기전에 대한 연구가 필요하리라 사료된다.

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