# Potential Endocrine Disrupting Effects of Phthalates in *In Vitro* and *In Vivo* Models

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## ABSTRACT

Thousands of new chemicals have been introduced to environment during last decades. Many of them and common consumer products have been shown to be the endocrine disrupting chemicals. One such chemical group is the phthalates, used in soft poly vinyl chloride (PVC) material and in a huge number of consumer products. The prevalence of these modern chemicals have a remarkable increase. Approximately 3.5 million tons of the main phthalate, di-(2-ethylhexyl) phthalate (DEHP), are produced annually worldwide and indeed, DEHP is considered a ubiquitous environmental contaminant. It has been demonstrated that high doses of phthalate can adversely affect adult and developing animals. In this review, we critically discuss the conclusions of recently original research papers and provide an overview of studies on reproductive disrupting effects of phthalates. In addition, we review the reproductive toxicity data of phthalates in some *in vitro* research and in both male and female reproductive systems in experimental and domestic animals. Finally, we point out some critical issues that should be addressed in order to clarify the implication of phthalates for human reproduction.

(Key words : phthalates, endocrine disruption, reproductive system, *in vivo*, *in vitro*)

## INTRODUCTION

Phthalates, dialkyl- or alkyl/aryl-esters of phthalic acid, are industrial chemicals used primarily as plasticizers to impart flexibility to polyvinylchloride plastics. They are present in a wide variety of products, including building materials, food packaging, clothing, toys and medical devices. In addition, some other phthalates are used as additives in cosmetics, pharmaceuticals, lubricant oils and solvents (Kavlock *et al.*, 2002). Each of these compounds has different levels of toxicity and they are known to cause reproductive disorders (Fabjan *et al.*, 2006). Approximately 3.5 million tons of the main phthalate, di-(2-ethylhexyl) phthalate (DEHP), are produced annually worldwide (Bornehag *et al.*, 2004) and indeed, DEHP is considered a ubiquitous environmental contaminant (Wams, 1987). Among all types of phthalates, DEHP is considered to be one of the most potent compounds causing adverse effects on reproduction and development in animal studies. DEHP is a priority pollutant in several countries; annual production amounts to 3~4 million tons and approximately 95% of them is used as a plasticizer in polyvinylchloride (PVC). DEHP is emitted to the environment during the production of plastics and plastic products, during their use and after disposal (Wams, 1987).

The effects of DEHP in the reproductive system have been considered of special relevance due to their recognized activity as endocrine disruptors (Latini *et al.*, 2004). The possible exposure of many industrial chemicals and pesticides to human and animals has been a growing concern over the last decade for both the scientific community and the general public. Several studies have suggested that these environmental contaminants could adversely affect reproductive functions in a variety of vertebrates (Foster *et al.*, 2001). DEHP is one of the most abundantly used phthalates and has been shown to induce developmental and reproductive toxicity in rodent models. The reproductive toxicity of DEHP in the rat and mouse has been characterized by reduction in fertility, litter size, sperm density and motility, and ovarian and testicular weights (Arcadi *et al.*, 1998). Thus in this review, we critically discuss the conclusions of recently original research papers and provide an overview of studies on reproductive disrupting effects of phthalates. In addition, we review the reproductive toxicity data of phthalates in some *in vitro* research and in both male and

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female reproductive systems in experimental and domestic animals. Finally, we point out some critical issues that should be addressed in order to clarify the implication of phthalates for human reproduction.

### 1. Exposure to Phthalate

The diesters of benzene-1,2-dicarboxylic (phthalic) acid, commonly known as phthalates, are a family of industrial compounds, primarily used as plasticizers to increase the flexibility of PCV products like: toys, vinyl flooring and electricity cables or medical devices with large amount of quantities (Latini *et al.*, 2006a). The main plasticizer used in PVC based medical devices is di-(2-ethylhexyl) phthalate (DEHP) which is not covalently bound to plastic matrix and can easily reach out to environment, thus becoming ubiquitous environmental pollution. Phthalates are used as solvents or fixing agents in perfumes, body lotions and other cosmetics (Bosnir *et al.*, 2003; Latini *et al.*, 2006b; Wittassek and Angerer, 2008). Globally, more than 18 billion pounds of phthalates are used each year. The most commonly used phthalate is DEHP and annual production volume of DEHP alone has been estimated at 2 million tons (Blount *et al.*, 2000). In spite of short halftime in the organism, the compounds or their metabolites have been detected in urine in more than 95% of men and women that have been investigated (Wittassek *et al.*, 2007).

Human are exposed to phthalates by multiple route like oral (phthalate-contaminated food, water and other liquids and in children through mouthing of toys and teethers) or dermal (cosmetics and other personal care products). Exposure can also be via inhalation; phthalates release from PVC, nail polish, hair spray, and other phthalate-containing products (Swan 2008). DEHP exposure in children has been changing in recent years. Although DEHP is no longer used in toys for children under the age of three in the EU (EU decision 1999/815/EG, renewed August 2003), as well as in the US and Canada regarding toys intended for mouthing (nipples, teething rings, pacifiers, rattles), however it is still found in toys for older children (Kavlock *et al.*, 2002).

Humans are constantly exposed to phthalates through oral, dermal and inhalation routes for example dermal exposure via clothes and cosmetics or oral via medical equipment (Wormuth *et al.*, 2006). Many endocrine disruptors are persistent in the environment and accumulate in fat tissue, and the lifespan of phthalates does not exceed 36 hours in the body. In human, 75% of DEHP ingested is metabolized and excreted in urine within 2 days (Koch *et al.*, 2005). Nevertheless, phthalates are widespread in the environment that humans are largely exposed. According to a study published in 2003, 12% of German population has a daily intake of DEHP that exceeds European recommendations (Koch *et al.*, 2003) .

### 2. Effects of Phthalate in *In-Vitro*

Phthalate plasticizers include butyl benzyl phthalate (BBP), dicyclohexyl phthalate (DCHP), diethyl phthalate (DEP), 2-ethylhexyl phthalate (DEHP), and di-n-butyl phthalate (DBP). They are made abundantly by humans, and suspected as an environmental chemical disrupting endocrine system (Harris *et al.*, 1997; Wieslander *et al.*, 1999). They have been reported to have estrogenic activities which mimic endogenous estrogen (Harris *et al.*, 1997; Harrison *et al.*, 1997; Moore 2000). There are numerous studies demonstrated that phthalates showed an estrogenic activity in several *in-vitro* tests: MCF7 cell proliferation, estrogen receptor (ER) binding in the rat uterus, and yeast transfected with human ER gene construct (Harris *et al.*, 1997; Jobling *et al.*, 1995; Parveen *et al.*, 2008). The suspected compounds can be detected quickly by these *in-vitro* tests. An E-screen method using the MCF-7 breast cancer cell line is one of the most sensitive methods for assessing the estrogenic activity of several phthalates (Soto *et al.*, 1995). DEHP at a concentration ($10^{-5}$ M) induced an increase in proliferation of MCF-7 cell. Di-n-alkyl phthalates like BBP, DBP and DEHP have estrogenic activity that mimics the steroid A ring of receptor binding modes of di alkyl phthalates (Asai *et al.*, 2000).

Recently, biomarkers have been imparted to assess the estrogenicity of endocrine disruptors (EDs) especially at low concentrations. The biomarkers response to EDs could be of mainly use for understanding the modes of these compound actions. Furthermore, the measurement of biomarker genes response provides a very sensitive an powerful tool to identify estrogenic compounds in the environment (Choi and Jeung, 2003). These include pS2, MUC1, androgen receptor, progesterone receptor, ER, lactoferrin, vitellogenin, cathepsin B (Heppell *et al.*, 1995; Ren *et al.*, 1997) and calbindin-9k (CaBP-9k)(Choi and Jeung, 2003; Dang *et al.*, 2007). CaPB-9k is a vitamin D-dependent calcium-binding protein, which belongs to a group of intracellular proteins that bind to calcium with high affinity and is localized in the mammalian intestine and uterus. (Kumar *et al.*, 1989). Jochen Reinsberg *et al* (Reinsberg *et al.*, 2009) reported that FSH-, hCG- and 8-Br-cAMP-stimulated estradiol production of granulosa cells was suppressed by MEHP in a dose-

dependent manner. Additionally, aromatase activity and mRNA levels were decreased in granulosa cells cultured with MEHP. MEHP is a specific inhibitor of estradiol production in human granulosa cells with a post-cAMP site of action.

In the previous studies, we suggested that CaBP-9k mRNA and protein expression might be a novel biomarker for estrogenic compounds in the uterus of immature rats. Therefore, in the current study, we assessed the estrogenic activity of diverse phthalates using *in-vitro* (E-screen test) models related to CaBP-9k mRNA and protein expression, and compare their estrogenic activity with other potential estrogenic chemicals. Our results showed that DEHP significantly stimulated MCF-7 cell proliferation at high concentration ($10^{-4}$) compared to vehicle (Hong *et al.*, 2005).

### 3. Effect of Phthalate on Male Reproductive System

Recently, many toxicity studies showed that exposure to certain phthalates resulted in severe disorders on the developing male rat reproductive system. DEHP-induced reproductive toxicity was reported during the perinatal period (Andrade *et al.*, 2006a; Gray *et al.*, 2000). Male offspring rats exposed in utero or during lactation to high phthalate doses (e.g. 750 mg DEHP/kg/day or 500 mg DBP/kg/day) showed reproductive tract abnormalities compatible with disruption of androgen-dependent development and impaired testicular function (Andrade *et al.*, 2006a; Gray *et al.*, 2000; Moore *et al.*, 2001; Nagao *et al.*, 2000). The phenotypic alterations appeared in male offspring including cryptorchidism, hypospadias (ectopic opening of the urethra), atrophy or agenesis of sex accessory organs, testicular injury, reduced daily sperm production, delayed preputial separation, permanent retention of nipples and decreased (feminized) anogenital distance. (Christiansen *et al.*, 2009; Vo *et al.*, 2009). Unlike other antiandrogens, which act by binding to the androgen receptor, phthalates disrupt the development of androgen-dependent structures mainly by inhibiting the fetal testicular testosterone biosynthesis (Mylchreest *et al.*, 2002; Parks *et al.*, 2000). This effect is mediated by changes in gene expression of enzymes and proteins involved in testosterone production by fetal Leydig cells (Lehmann *et al.*, 2004; Liu *et al.*, 2005). The AGDs of male rat pups were significantly shorter in the group of dams treated with 750 mg/kg DEHP compared with a control (Lin *et al.*, 2009).

Serum testosterone and LH concentration levels in male fetus at gestation day (GD) 21 and offspring rats at postnatal day (PND) 63 were significantly reduced at a high dose (500 mg/kg BW/day) of DEHP compared to a control group. The AGD indexes of male offspring at PND 63 were significantly decreased in the groups exposed *in utero* to the intermediate dose of DEHP (100 mg/ kgBW/day) (Vo *et al.*, 2009). A high dose of DEHP followed maternal exposure induced an increase in a number of nipples of male offsprings (Vo *et al.*, 2009). Especially, at the PND 63 some nipples and/or areole were regressed at the PND 63 (data not shown). Hypospadias were shown in all male rats exposed to DEHP (500 mg/kg BW/day). Remarkably, cryoptorchidism was increased in response to maternal DEHP exposure (500 mg/kg), with 17.4% of male rats exhibiting undescended testes. The descending time and place of testes in the scrotum were significant differences in the highest dose of DEHP (500 mg/kg) but not found any significant changes in lower doses.

Vo *et al.* (2009) showed that a high dose of DEHP (500 mg/kg BW/day) exposed to immature male rats statistically reduced the reproductive organ weights including testes, prostate and seminal vehicle. Furthermore, this high dose also significantly decreased AGD when compared with a control group. Interestingly, there was a significant decrease in the level of testosterone when immature rats were exposed to all doses of DEHP (10, 100 and 500 mg/ kg BW/day), however no significant changes of LH were shown at any doses. Histological results showed that degeneration of Leydig cells and disorders of germ cells in the reproductive tract were noted in response to all doses of DEHP. Shirota M *et al* (Shirota *et al.*, 2005) indicated that in utero exposure to DEHP (1000 mg/kg BW/day) caused the dilatation and atrophy of seminiferous tubules in rats. Additionally, exposure to DEHP (500 mg/kg BW/day) may result in the abnormalities of cell morphology in which multinucleated germ cells were observed in seminiferous cords.

### 4. Effect of Phthalate on Female Reproductive System

Unlike to males, it is generally thought that the female reproductive system is much less sensitive to phthalates. Nevertheless, recent evidence suggests that phthalates can also induce adverse responses in females following pre- and postnatal exposure (Grande *et al.*, 2006; Grande *et al.*, 2007; Gray *et al.*, 2006). Initial studies demonstrated that the ovary is a target site for DEHP. It was reported that a high dose of DEHP (2,000 mg/kg/day) resulted in prolonged estrous cycles, and reduced serum estradiol levels and absence of ovulation in adult rats (Davis *et al.*, 1994). Long term exposure to DEHP resulted in continuous diestrous with decreased serum estradiol and fo-

llicle stimulating hormone (FSH), pituitary FSH and luteinizing hormone (LH) (Hirosawa *et al.*, 2006). Several fertility studies with crossover breeding have demonstrated that active phthalates-like DEHP and DBP can reduce the fertility of rats and mice through male and female-mediated effects (Gray *et al.*, 1999; Lamb *et al.*, 1987). According to Gray *et al.*, oral administration of dibutyl phthalate (DBP) to female Long Evans rats from weaning, through puberty, mating, and gestation disrupts pregnancy maintenance at dose levels similar to those that affect testis function in male rats. This study also showed DBP-induced midpregnancy abortions which were associated with increased progesterone and decreased estradiol production (Gray *et al.*, 2006).

Recently, there were several studies about postnatal consequences of in utero and lactational DEHP exposure in rats (Grande *et al.*, 2006; Grande *et al.*, 2007). The results of Grande *et al.*, demonstrated that DEHP exposure resulted in a delay in the age of puberty onset (vaginal opening) in female offspring at doses of 15, 45, 135 and 405 mg/kg/day (Grande *et al.*, 2007). Interestingly, when male littermates were evaluated for preputial separation, a marker of puberty onset in male rats, a significant delay was observed at the same doses causing delayed vaginal opening in females (Andrade *et al.*, 2006a). Nevertheless, during adulthood, female offspring exposed in utero and during lactation did not show any sign of disturbed reproductive function, with the exception of an increase in the incidence of tertiary atretic follicles in animals exposed to the highest dose tested (405 mg/kg/day) (Grande *et al.*, 2007). No adverse changes were detected in estrous cyclicity, serum estradiol and progesterone concentrations or reproductive organ weights. This is in contrast with the results obtained with adult male offspring, which showed impaired testicular function and reproductive tract abnormalities at doses as low as 15 and 5 mg DEHP/kg/day, respectively (Andrade *et al.*, 2006b). In summary, these results indicate that although changes were seen in both young male and female offspring exposed in utero and during lactation to similar doses, adult female offspring appear to be less sensitive to persistent effects on the reproductive system than their adult male counterparts.

### 5. Effects of Phthalate in Mammal Animals

DEHP is a lipophilic compound and, therefore, it is not chemically combined to poly vinyl chloride (PVC) therefore DEHP can be released from plastic (Takehisa *et al.*, 2005). Experimental exposure to DEHP has mainly been analyzed in rodent models (Calafat *et al.*, 2006). However, knowledge of animal exposure to DEHP is important for understanding the potential risk to animal health as well as the risk to human health, as the animal derived products represent one of the most important sources of human exposure to many organic pollutants. Nevertheless, until very recently, studies of plasma levels in domestic animals have been limited (Rhind *et al.*, 2005). Mono (2-ethylhexyl) phthalate at doses 70 and 100 $\mu$M resulted in a significantly higher rate of bovine oocytes remained at the germinal vesicle stage compared to control (Anas *et al.*, 2003).

## CONCLUDING MARKERS

Phthalates are widespread environmental contaminants and some of them have shown significantly reproductive and developmental toxicity in animals as well as in some cell lines. Recent studies have reported the associations with pre- and postnatal phthalate exposure in male reproductive systems. This may be a consequence of the anti-androgenic activities of these compounds. Basically, high doses of phthalates are required to adversely affect the male and female reproductive system in adults with the testis and ovary being considered as the crucial target organs.

Although most reproductive tract abnormalities induced by phthalates occur at doses well above the estimated intake of the general population. Furthermore, recent experiment results indicate that biological changes can also be induced at low doses, human relevant doses and that different active phthalates can have cumulative effects. Nevertheless, uncertainties in the epidemiological data base, difficulties in animal to human extrapolations and lacks of knowledge on the significance of low-dose effects for human health preclude a better understanding of the real risks for humans. Further investigation on possible cumulative effects of different active phthalates and low-dose effect should be concerned.

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