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Anti-Androgenic Activity of Phthalate Esters (Di(2-ethylhexyl) Phthalate, Di(n-butyl) Phthalate, and Butylbenzyl Phthalate) in the Rodent 10-day Hershberger Assay using Immature Castrated Male Rats

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ABSTRACT. The rodent Hershberger assay is considered as a potential short term *in vivo* screening method for the detection of androgenic or anti-androgenic compounds. The objective of this study was to evaluate the anti-androgenic activities of di(2-ethylhexyl) phthalate (DEHP), di(n-butyl) phthalate (DBP), and butylbenzyl phthalate (BBP). A 10-day Hershberger assay was performed using immature Sprague-Dawley male rats castrated at 6 weeks of age. Testosterone propionate (TP, 0.4 mg/kg/day) was administered s.c. to castrated male rats and followed by flutamide (1, 5, 10, or 20 mg/kg/day) treatment for 10 days by oral gavage. Similarly, DEHP, DBP, or BBP were also administered by oral gavage at 250, 500, or 1000 mg/kg/day after TP (0.4 mg/kg/day) administration. As expected, flutamide significantly inhibited the TP-induced re-growth of seminal vesicles, ventral prostate, and *Levator ani plus bulbocavernosus muscles* (LABC) at 1 mg/kg/day and above, and Cowper's glands and glans penis at 5 mg/kg/day and above. DEHP significantly ($p < 0.05$) decreased the seminal vesicles, ventral prostate, LABC and Cowper's glands weights at 1000 mg/kg/day. BBP at 1000 mg/kg/day significantly inhibited TP-induced re-growth of the LABC in the immature castrated male rats, whereas ventral prostate, seminal vesicles, and Cowper's glands weights were unaffected. In contrast to DEHP, DBP did not affect accessory sex organ weights at any concentration. Body weights, combined adrenal glands, and kidney weights were not affected, but liver weights were significantly increased at high dosages in the DEHP, DBP, and BBP treatment groups. Our observations strongly suggest that DEHP acts as an androgen antagonist at the high dose (i.e., 1000 mg/kg/day).

Keywords: 10-day Hershberger assay, Antiandrogen, Flutamide, Di(2-ethylhexyl) phthalate, Di(n-butyl) phthalate, Butylbenzyl phthalate.

INTRODUCTION

Potential reproductive and developmental disorders associated with certain phthalate esters, particularly di(2-ethylhexyl) phthalate (DEHP), di(n-butyl) phthalate (DBP) and butyl benzyl phthalate (BBP) are viewed with concern, due to their abilities to affect the reproductive organ abnormalities via estrogen- or androgen-mediated mechanisms (Gray *et al.*, 2001; Moore, 2000; Parks *et al.*, 2000). In general, phthalate esters are used predominantly as plasticizers in polyvinyl chloride (PVC) products and as solvent of the painting inks used in food packaging or in certain personal care products. These phthalates do not persist in the environment, and are rapidly metabolized and eliminated in the environment and in exposed animals (Group, 1986; Foster *et al.*, 1983). However, considerable uncertainty exists regarding the effects of these phthalate esters during development. Recently, regulatory action on the usage of certain phthalate esters in toys for infants was

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prompted at least in part by concern over their potential endocrine activities (Bouma and Schakel, 2001; Wilkinson and Lamb, 1999).

In fact, several studies have demonstrated that phthalate esters may disrupt the endocrine system, and produce severe alterations in male reproductive organ development (Gray *et al.*, 1999; Mylchreest *et al.*, 1999). In addition, some phthalate esters (DEHP, DBP, and BBP) have been reported to have estrogenic activity in some *in vitro* assay systems (Harris *et al.*, 1997; Jobling *et al.*, 1995; Zacharewski *et al.*, 1998). Moreover, DBP and BBP were found to induce estrogen responsive element (ERE)-regulated reporter gene and to stimulate human breast cancer cell proliferation (Jobling *et al.*, 1995). For these reasons, it has been proposed that the estrogenic activities of some phthalate esters are closely associated with reproductive organ disorders observed in rodents administered with these agents during critical developmental stages (Sharpe *et al.*, 1995; Wine *et al.*, 1997). However, no phthalate ester was found to induce an adverse effect associated with estrogenic activity *in vivo* (Wine *et al.*, 1997; Mylchreest *et al.*, 1998; Gray *et al.*, 1999). In fact, many studies have found that the abnormality patterns observed for phthalate esters are indicative of anti-androgenic effects, in particular a significant effect was observed for reproductive tract sexual differentiation (Mylchreest *et al.*, 1998; Gray *et al.*, 1999). Moreover, DBP and DEHP induced reproductive organs disorders, which were mediated by their mono-substituted phthalate ester metabolites (Dostal *et al.*, 1998; Foster *et al.*, 1983). However, Maness *et al.* (1998) found that neither DBP nor DEHP, nor their metabolites MBP and MEHP, respectively, were able to interact directly with human androgen receptor (AR) *in vitro*. Furthermore, marked differences in responses were observed between phthalate esters (DEHP and DBP) and the pure androgen receptor (AR) antagonist, flutamide (Mylchreest *et al.*, 1998; Gray *et al.*, 1999).

However, no study has been conducted to determine the potential anti-androgenic activities of DEHP, DBP, and BBP using the *in vivo* study. Therefore, the aim of the present study was to evaluate the anti-androgenic effects of these phthalate esters in the 10-day Hershberger assay using castrated immature male rats. Recently, the Hershberger assay has been used to detect chemicals with AR mediating activity, as it was designed to detect the androgenic or anti-androgenic activities of test compounds (Ashby and Lefevre, 2000a; Gray *et al.*, 1998; Kim *et al.*, 2002; O'Connor *et al.*, 1999). The advantages of this assay are that it is straightforward to perform, rapid, and relatively specific for the detection of androgenic or anti-androgenic compounds. In the present study, the 10-day Hershberger assay using immature castrated male rats was used to evaluate if DEHP, DBP, and BBP interfere with AR-mediated mechanisms *in vivo*.

MATERIALS AND METHOD

Chemicals

Testosterone propionate (TP) was purchased from TNO (Zeist, Netherlands). Flutamide (Purity: 99%, Catalog No. F9397), DEHP (Purity >99%, Catalog No. 525154), DBP (Purity >98%, Catalog No. 524980), and BBP (Purity >97%, Catalog No. 513458) were purchased from the Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI), respectively. Chemicals were used as supplied; all were stored in glass containers at room temperature.

Animal housing and castration

Sprague-Dawley male rats (4 weeks of age, Charles River Laboratories) were obtained from the Korean Food and Drug Administration Animal Resource Laboratory (Seoul, Korea) and housed under specific pathogen free (SPF) conditions. All rats were housed in clear polycarbonate cages for 2 weeks prior to castration and were maintained under a 12 h light-dark cycle at 50 ± 10% RH and 23 ± 2°C. Prior to the experiment, all animals were checked for overt signs of illness and only healthy animals were selected. During the study, all animals were provided filtered tap water and a Certified Rodent *LabDiet* (Purina, USA). Food and water were available *ad libitum*. The procedure used for castration was as described by the OECD protocol (OECD, 2001). All animals were housed in polycarbonate cages and acclimatized to laboratory conditions over at least 2 weeks before castration, which was performed at 6 weeks old. Animals were castrated via a midline incision, and test chemicals were administered from a minimum of 8 days later to allow complete recovery. Animals were allocated to the various body weight-matched treatment groups by random sorting. The body weight variations per group were within mean ± 5 g.

Study groups

Flutamide (1, 5, 10, or 20 mg/kg/day) as a positive control was administered by oral gavage within 15 min after TP (0.4 mg/kg/day) was administered s.c. to castrated male rats. DEHP, DBP, or BBP (all at 250, 500, or 1000 mg/kg/day) were administered by oral gavage for 10 days, and then TP (0.4 mg/kg/day) was administered by s.c. injection within 15 min. The vehicle control (VC) group was administered corn oil. The maximum

volume administered per animal was approximately 0.5 ml/kg for s.c. injection and 4 ml/kg for oral gavage, respectively. Dosage levels were adjusted for body weight changes.

Clinical signs, body weight, and organs weight

Through the test period, each animal was observed at least once a day. On working days, all cages were checked in the morning and afternoon for dead or moribund animals. All abnormalities were recorded. Individual body weights were recorded prior to start of treatment (to the nearest 0.1 g), on each day of treatment and prior to necropsy. After necropsy, sex accessory tissues were removed and weighed (to the nearest 0.1 mg) without blotting, and the excised tissues were trimmed of any fat. A standard operating procedure was followed for the excision of sex accessory tissues, specifically standard excision procedures were used and particular care was taken to prevent variations in tissue fluid losses during processing. The weights of following accessory sex tissues were measured: ventral prostate, seminal vesicle (together with the coagulating glands), LABC, Cowper's glands, and glans penis. In addition, livers, adrenal glands, and kidneys were also excised, and their weights measured.

Statistical analyses

All values are expressed as means ± SE (n = 6 animals). Statistical analysis was performed using one-way analysis of variance (ANOVA) and the Dunnett's test (*SigmaStat*, Jandel Scientific Co., Germany). Significant differences between groups were determined by two-way ANOVA and $p < 0.05$ was considered statistically significant.

RESULTS

The anti-androgenic effects of flutamide on TP-induced accessory sex organ re-growth are shown in Fig. 1. As expected, flutamide significantly inhibited TP-induced accessory sex organ re-growth in a dose-related manner. Statistically significant differences in ventral prostate, seminal vesicle, LABC, and Cowper's glands weights were observed at flutamide doses of above 1.0 mg/kg/day. Seminal vesicle weights were significantly reduced at doses of flutamide 1.0 (58% of control), 5.0

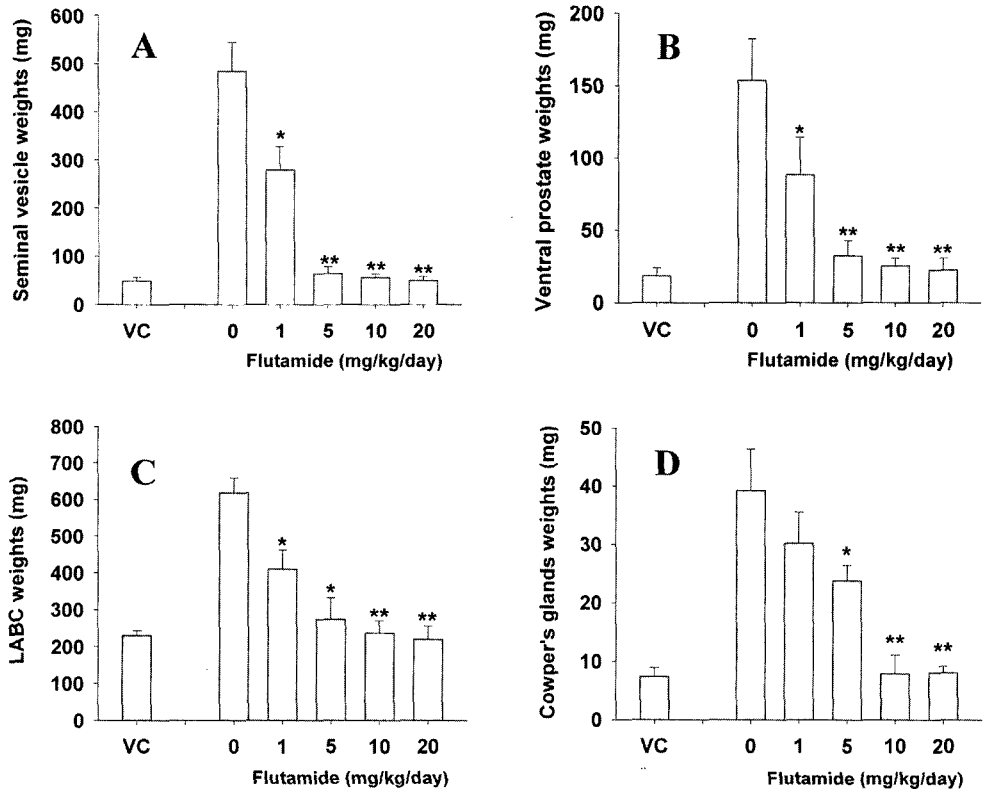

Fig. 1. Absolute tissue weights from immature castrated Sprague-Dawley rats given 10 consecutive daily treatments of testosterone propionate, commencing 8 days after castration. A; Seminal vesicle, B; Ventral prostate, C; *Levator ani plus bulbocavernosus muscles* (LABC), D; Cowper's glands. *p < 0.05 and **p < 0.01 versus the vehicle control.

(13% of control), 10 (12% of control), or 20 mg/kg/day (10% of control) versus the control; ventral prostate by 57, 21, 17, and 15% versus the controls; and LABC by 66, 44, 38, and 36% versus the controls, respectively. In addition, Cowper's glands weights were also significantly reduced at flutamide doses of ≥ 5 mg/kg/day (Fig. 1).

DEHP caused a statistically significant decrease in seminal vesicle (65% of control), ventral prostate (73% of control), and LABC (84% of control) weights but only at the high dose of 1000 mg/kg/day, but body weights, kidney, and adrenal weights were unaffected (Table 1). However, liver weights were significantly increased at 500 (136% of control) and 1000 mg/kg/day (151% of control) (Table 1). In contrast to DEHP, DBP did not affect the TP-induced re-growth of accessory sex organs at any concentration, although DBP at 1000 mg/kg/day also markedly increased liver weights in a dose-dependent manner ($p < 0.05$) (Table 2). BBP at 1000 mg/kg/day caused a statistically significant decrease in LABC weights (83% of control), whereas ventral prostate, seminal vesicle, and Cowper's glands weights were unaffected at any concentration (Table 3). As was found for DEHP and DBP, BBP significantly increased liver weights significantly at the dose of 1000 mg/kg/day (Table 3).

Table 1. Hershberger assay response to di(2-ethylhexyl) phthalate in immature Sprague-Dawley male rats by 10 days consecutive treatments

Organ weight	Vehicle control[b]	Testosterone propionate[a] 0.4 mg/kg/day			
		Control 0 mg/kg	DEHP 250 mg/kg	DEHP 500 mg/kg	DEHP 1000 mg/kg
Initial B.W. (g)	243.2 ± 8.0[c]	243.5 ± 9.9	244.2 ± 7.1	243.7 ± 5.9	243.8 ± 6.3
Final B.W. (g)	304.8 ± 8.6	323.7 ± 11.6	322.8 ± 14.4	324.2 ± 16.7	312.7 ± 8.2
Liver (g)	12.4 ± 0.7	13.5 ± 0.6	16.4 ± 0.8	18.4 ± 1.7*	20.4 ± 1.9*
Kidney (g)	2.27 ± 0.20	2.12 ± 0.06	2.17 ± 0.03	2.16 ± 0.03	2.17 ± 0.02
Adrenals (mg)	48.7 ± 1.6	51.2 ± 3.6	51.5 ± 7.8	56.2 ± 5.5	53.4 ± 7.3
Seminal vesicle (mg)	50.0 ± 6.4	661.6 ± 73.2	555.9 ± 59.7	502.6 ± 35.4	432.5 ± 45.9*
Ventral prostate (mg)	17.8 ± 1.5	173.8 ± 14.1	173.4 ± 51.6	159.4 ± 18.9	127.5 ± 16.0*
LABC (mg)	195.4 ± 24.5	563.1 ± 56.4	542.1 ± 43.6	552.7 ± 45.2	470.5 ± 30.8*
Cowper's glands (mg)	6.8 ± 2.0	51.0 ± 6.8	45.1 ± 7.4	44.6 ± 4.7	34.2 ± 8.8*
Glans penis (mg)	40.8 ± 5.4	96.0 ± 1.7	100.0 ± 9.8	96.8 ± 3.5	92.8 ± 5.7

[a]Castrated immature male rats were administered with testosterone propionate (0.4 mg/kg/day) by subcutaneous injection and di(2-ethylhexyl) phthalate by oral gavage for 10 days. One day after the final treatment, the accessory sex organs were removed carefully and weighed separately.
[b]Vehicle control received corn oil containing 2.5% ethanol.
[c]Data are presented as mean ± SD (n = 6), Significantly different from control at *$p < 0.05$.

Table 2. Hershberger assay response to di(n-butyl) phthalate in immature Sprague-Dawley male rats by 10 days consecutive treatments

Organ weight	Vehicle control[b]	Testosterone propionate[a] 0.4 mg/kg/day			
		Control 0 mg/kg/day	DBP 250 mg/kg	DBP 500 mg/kg	DBP 1000 mg/kg
Initial B.W. (g)	244.1 ± 8.4[c]	243.2 ± 6.8	243.6 ± 3.8	243.8 ± 8.4	243.1 ± 10.2
Final B.W. (g)	308.5 ± 8.1	328.3 ± 15.6	331.6 ± 15.4	334.3 ± 15.3	323.5 ± 17.7
Liver (g)	11.9 ± 0.5	13.1 ± 1.2	14.4 ± 0.9	14.9 ± 0.8	16.0 ± 1.4*
Kidney (g)	1.97 ± 0.14	2.22 ± 0.29	2.21 ± 0.16	2.24 ± 0.09	2.21 ± 0.14
Adrenals (mg)	61.9 ± 8.4	54.0 ± 6.7	56.1 ± 6.6	55.0 ± 4.2	49.4 ± 6.8
Seminal vesicle (mg)	51.0 ± 8.2	450.8 ± 59.3	454.3 ± 67.5	523.2 ± 58.0	458.2 ± 52.8
Ventral prostate (mg)	17.8 ± 2.5	153.6 ± 21.5	152.3 ± 39.6	191.5 ± 10.5	141.7 ± 18.3
LABC (mg)	208.4 ± 31.2	548.6 ± 37.0	519.7 ± 60.7	580.1 ± 45.2	524.2 ± 73.2
Cowper's glands (mg)	6.3 ± 1.7	34.9 ± 6.8	40.8 ± 9.8	41.6 ± 5.9	38.9 ± 8.1
Glans penis (mg)	44.3 ± 7.5	85.6 ± 5.5	83.1 ± 12.5	78.5 ± 1.9	74.9 ± 7.0

[a]Castrated immature rats were administered with testosterone propionate (0.4 mg/kg/day) by subcutaneous injection and di(n-butyl) phthalate by oral gavage for 10 days. One day after the final treatment, the accessory sex organs were removed carefully and weighed separately.
[b]Vehicle control received corn oil containing 2.5% ethanol.
[c]Data are presented as mean ± SD (n = 6), Significantly different from control at *$p < 0.05$.

Table 3. Hershberger assay response to butylbenzyl phthalate in immature Sprague-Dawley male rats by 10 days consecutive treatments

Organ weight	Vehicle control[b]	Testosterone propionate[a] 0.4 mg/kg/day			
		Control 0 mg/kg	BBP 250 mg/kg	BBP 500 mg/kg	BBP 1000 mg/kg
Initial B.W. (g)	236.6 ± 6.8[c]	237.3 ± 12.7	234.2 ± 13.5	237.5 ± 8.1	236.3 ± 9.5
Final B.W. (g)	303.2 ± 9.4	315.5 ± 17.4	317.8 ± 20.2	322.6 ± 16.0	322.6 ± 15.2
Liver (g)	11.9 ± 0.2	12.4 ± 0.7	13.5 ± 1.5	14.8 ± 0.5	16.5 ± 1.7*
Kidney (g)	2.07 ± 0.15	2.10 ± 0.08	2.24 ± 0.21	2.31 ± 0.13	2.45 ± 0.17
Adrenals (mg)	50.9 ± 9.8	52.3 ± 5.9	47.2 ± 8.4	50.4 ± 6.2	46.5 ± 6.3
Seminal vesicle (mg)	50.2 ± 9.3	524.1 ± 66.2	449.0 ± 50.0	481.5 ± 52.9	496.4 ± 46.7
Ventral prostate (mg)	15.7 ± 1.5	163.4 ± 17.6	193.1 ± 22.4	163.0 ± 20.9	163.5 ± 15.5
LABC (mg)	207.6 ± 27.5	588.0 ± 68.3	556.9 ± 60.1	503.5 ± 43.9	486.4 ± 34.2*
Cowper's glands (mg)	9.7 ± 2.1	40.9 ± 7.8	46.3 ± 7.1	39.5 ± 6.7	35.9 ± 4.8
Glans penis (mg)	42.3 ± 5.9	80.8 ± 5.4	80.9 ± 7.1	71.5 ± 8.6	77.8 ± 9.6

[a]Castrated immature rats were administered with testosterone propionate (0.4 mg/kg/day) by subcutaneous injection and butylbenzyl phthalate by oral gavage for 10 days. One day after the final treatment, the accessory sex organs were removed carefully and weighed separately.
[b]Vehicle control received corn oil containing 2.5% ethanol.
[c]Data are presented as mean ± SD (n = 6), Significantly different from control at *$p < 0.05$.

DISCUSSION

Recently, increasing evidence indicates that a large number of environmental chemicals may alter male sexual differentiation because of their anti-androgenic activity (Foster *et al.*, 2001; Gray *et al.*, 1999; Kelce and Wilson, 1997). Of these, some phthalate esters have been reported to cause abnormal sexual development and reproductive organ disorders by acting primarily as anti-androgens (Mylchreest *et al.*, 1998; Moore *et al.*, 2001). However, no study has been conducted to evaluate their anti-androgenic activities using the Hershberger assay. Therefore, we undertook this work to investigate the anti-androgenic mechanisms of DEHP, DBP, and BBP in a 10-day Hershberger assay using an immature castrated male rat model. Our results demonstrate that whereas DEHP showed weak anti-androgenic activity, DBP and BBP did not. Therefore, we find that certain phthalate esters may disrupt normal male reproductive organ development by acting as anti-androgens.

Numerous methodologies are available for assessing the endocrine disrupting effects of chemicals *in vivo* and *in vitro* (Ashby and Lefevre, 2000a; 2000b; Gray *et al.*, 1998; O'Connor *et al.*, 1999; 2002). And some of these methodologies have been used to evaluate the estrogenic or anti-androgenic activities of phthalate esters. In the case of phthalate esters, initial concerns that they are endocrine disruptors were raised because they were reported to have estrogenic activity in *in vitro* assay systems, such as, by competitive binding (Picard *et al.*, 2001), recombinant yeast screening (Harris *et al.*, 1997), and by the E-screen assay (Jobling *et al.*, 1995). However, phthalate esters, including DEHP and DBP, were also reported not to show any positive uterotrophic response (Zacharewski *et al.*, 1998). These workers investigated the estrogenic activities of eight phthalate esters by administering doses of 20, 200, or 2000 mg/kg/day to ovariectomized (OVX) rats. None of the phthalates were found to induce either absolute or relative uterine weight increases in immature rats, or vaginal epithelium cornification in mature rats. Furthermore, monoethylhexyl phthalate (MEHP) and monobutyl phthalate (MBP), the active metabolites of DEHP and DBP, respectively, did not interact with the ER *in vitro*. Consequently, their effects on the development of the reproductive system are unlikely to involve binding to the estrogen receptor (ER). These results are consistent with those reported by Mylchreest *et al.* (1998), who investigated the effect of DBP on the prenatal and early neonatal development of the reproductive tract. These findings indicate that DBP specifically impairs the androgen-dependent development of the male reproductive tract, which suggests that DBP is not estrogenic but anti-androgenic in rats at high doses. In this study, DBP was found to produce the same spectrum of effects elicited by flutamide in male offspring.

In contrast, although the effects of phthalate esters on the developing male reproductive tract are similar to those of flutamide, neither phthalates nor their primary metabolites interact with AR (Foster *et al.*, 2001), and some organ sensitivity differences were observed be-

tween phthalate and flutamide on male reproductive development (Mylchreest *et al.*, 1998). DBP (500 mg/kg/day) significantly affected the epididymis and vas deferens, but not seminal vesicles. These observations strongly suggest that DBP and other phthalate esters do not act as anti-androgens in rats. Our results are in accordance with most of the results of Mylchreest *et al.* (1998), with the exception that we found that DEHP may act as an anti-androgen in the 10-day Hershberger assay. In addition, phthalate esters are known to inhibit the synthesis of testosterone in male rats. When DEHP was administered at 750 mg/kg/day during the prenatal stage, testicular testosterone production, testicular and fetal testosterone levels were reduced to female levels during sexual differentiation (Parks *et al.*, 2000).

The reliability of the 10-day Hershberger assay protocol used in the present study has been previously described (Kang *et al.*, 2003). However, to validate our experimental protocol, flutamide was also evaluated in the present study. As expected, flutamide (1 mg/kg/day and above) significantly attenuated the TP (0.4 mg/kg/day)-induced re-growth of seminal vesicle, ventral prostate, and LABC. With regard to accessory sex organs sensitivity to flutamide, seminal vesicle and ventral prostate appeared to be more sensitive than LABC, Cowper's glands, and glans penis at flutamide doses of $\geq$ 5 mg/kg/day. Of the phthalate esters tested in the present study, only DEHP (1000 mg/kg/day) significantly reduced accessory sex organ weights. Similar to our results, Ashby and Lefvere (2000b) found that DBP (1000 mg/kg/day) produced no significant difference in Cowper's glands, LABC, seminal vesicle, and prostate weights in animals after 5 days of exposure. In contrast, 10 days of exposure to DBP (1000 mg/kg/day) significantly reduced LABC weights; however, the responsiveness of seminal vesicle, prostate, and Cowper's glands were highly variable.

The purpose of the Hershberger assay is to assess endocrine modulation without any systemic toxicity, because severe systemic toxicity interferes with normal endocrine function (Ashby and Lefevre, 2000b). For example, body weight suppression of more 15~20% can influence hormone concentrations and organ weights (O'Connor *et al.*, 1999, 2000). Several studies have reported that phthalate esters act as peroxisome proliferators, and thus induce hepatic microsomal enzymes, enlarge livers, and increase the risk of liver cancer (Lake *et al.*, 1984; Mitchell *et al.*, 1985). Howarth *et al.* (2001) found that male Wistar albino rats receiving diets containing DEHP alone or in combination with di(n-hexyl) phthalate (DnHP) showed markedly elevated liver weights. In the present study, although body weight change was not observed at the highest doses of the phthalate esters, liver weights were significantly increased versus the controls at 1000 mg/kg/day.

In conclusion, it is evident that the 10-day Hershberger assay is sensitive enough to detect anti-androgenic activity of phthalate esters. Our findings suggest that DEHP is an androgen antagonist, and that it is capable of disrupting reproductive organ development by acting either as an AR antagonist or via some other mechanisms.

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