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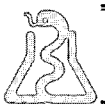

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Uterotrophic and Hershberger Assay for Butyl ρ-Hydroxybenzoic Acid

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ABSTRACT. Butyl ρ-hydroxybenzoic acid (butyl paraben, BP) is a homologous series of parabens and is widely used as a preservative in cosmetic and pharmaceutical products. The purpose of this study was to investigate the estrogenic/antiandrogenic activities of BP in animals. For that, we performed an uterotrophic assay and a Hershberger assay in rats. In uterotrophic assay, BP was administered subcutaneously to immature female SD rats (18 days old) for 3 consecutive days. The wet and dry uterus weights were significantly increased in the groups treated with BP in dose-dependent manner. In case of Hershberger assay, BP significantly reduced the weight of seminal vesicle of castrated rats. And other accessory organ/glands - prostate, Cowper's glands, bulbocavernosus muscle and glans penis were also slightly decreased. The results of this study suggested that BP showed estrogenic and anti-androgenic activities *in vivo*.

Keywords: Butyl ρ-hydroxybenzoic acid, Uterotrophic assay, Hershberger assay

INTRODUCTION

Many environmental pollutant chemicals have been shown, in recent years, to possess oestrogenic properties (Andersen *et al.*, 1999) and have been termed xenoestrogens or environmental estrogens. Xenoestrogens identified to form a bewildering array of structurally diverse chemicals which can be rather different from the natural estrogen 17β-estradiol (E_2), and which exert a range of estrogen agonist and antagonist properties in different assay systems. According to epidemiological data, a progressive decline in human male reproductive health and fertility may be associated with exposure to endocrine-disrupting chemicals (EDCs) in the environment those which mimic the action of natural estrogens (Sharpe *et al.*, 1995).

Although many natural and synthetic chemicals are in wide use and are ubiquitous in the environment, little is known about the potential risks to humans following exposure to known xenoestrogens, or their potential routes and levels of exposure.

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List of abbreviations: BP, butyl ρ-hydroxybenzoic acid; E_2, 17β-estradiol; TE, testosterone esters

Parabens are compounds with anti-bacterial and anti-fungal properties and are extensively used in food preservatives and in cosmetics due to their low toxicity, broad spectrum of activity, worldwide regulatory acceptance, biodegradability, low cost, and chemical stability (Elder, 1984). Among parabens, butyl ρ-hydroxybenzoic acid (BP) was shown to have potent xeno-estrogenic effect by *in vitro* and *in vivo* assay systems (Soto *et al.*, 1991; Routledge and Sumpter, 1997).

In this study, a 3-day uterotrophic assay using immature female rats and a 10-day Hershberger assay using catrated peripubertal male rats were performed to determine if BP has estrogenic or (anti)-estrogenic activities *in vivo*. To validate our experimental protocol, responses to reference chemicals were concurrently assessed as positive controls: E_2 for estrogenicity in uterotrophic assay, and testosterone esters (TE) for androgenicity in Hershberger assay.

MATERIALS AND METHODS

Chemicals

BP was supplied from Clariant, Co. LTD., UK.

Animals

Immature female (12 days old) and mature male (5 weeks old) SD rats were obtained from Orient, Co. LTD., Korea. The experiment started after 1 week accli-

matization. Drinking water and pellet rodent diet were available *ad libitum*. All male rats were castrated via the scrotum under ether anesthesia at 5 weeks of age, and allowed 7 days for complete recovery from surgical stress.

Uterotrophic assay

The protocol for the immature rat uterotrophic assays was as described previously (Odum *et al.*, 1997). BP and reference chemicals were subcutaneously administered for 3 consecutive days to 18 day old rat. All chemicals were dissolved in corn oil. The E_2 (0.04 mg/kg/day) was administered as a positive control. A total dosing volume of 5 ml/kg body weight was used initially. But this was increased to 10 ml/kg because of low solubility of BP. The dose levels of BP were 400, 800, 1000 and 1200 mg/kg/day for immature uterotrophic assay. Animals were killed by decapitation 24 h after the final dose. The uterus was then dissected and weighed (blotted weight) after careful trimming to remove fat and other contiguous tissue. Uterine dry weight was also determined by drying the uteri at 50°C for overnight before reweighing.

Hershberger assay

For assessment of anti-androgenicity of BP, 0.8 mg/kg of TE (Duratestone®) was concurrently administered. BP was administered to castrated or TE-treated castrated rats by s.c. injection for 10 consecutive days. The dose levels of BP were 800, 1000 and 1200 mg/kg/day. One day after the final administration, rats were decapitized and then the sexual accessory glands/tissues (prostate, seminal vesicles with coagulating glands, bulbocavernosus muscle, glans penis and Cowper's glands) as well as liver were dissected.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). The data were expressed as mean ± standard deviation (SD). The criterion for significance was $P < 0.01$.

Table 1. Effect of BP on body weights in immature female rats for estrogenicity

Chemicals	Doses (mg/kg/day)	Initial body weight (g)	Final body weight (g)
Vehicle	0	35.9 ± 1.6[a]	56.1 ± 2.3
BP	400	36.1 ± 1.4	54.4 ± 1.6
	800	35.6 ± 2.2	54.5 ± 4.6
	1000	34.9 ± 2.7	53.6 ± 4.1
	1200	34.5 ± 3.1	53.7 ± 5.1
E_2	0.4	34.0 ± 1.7	52.9 ± 3.4

[a]Values are Mean ± SD (n = 10).
Immature female rats were administered butyl ρ-hydroxybenzoic acid (BP) by s.c. injection for 3 consecutive days. E_2, 17β-estradiol.

RESULTS

Uterotrophic assay

BP was tested in immature rat uterotrophic assays by subcutaneous route. Body weights are shown in Table 1. No clinical abnormalities were observed in any of groups, and body weight increased normally in normal group. The result of the immature rat uterotrophic assays on BP is shown in Table 2. BP significantly increased both blotted and dry uterine weights with dose-dependency ($P < 0.01$). The relative weight changes of uterus were essentially the same as absolute weight changes. The treatment with E_2, the reference chemical, dramatically increased the absolute and relative weight of both blotted and dry uterine weight.

Hershberger assay

TE (0.8 mg/kg/day) did not induce any clinical signs and any changes in body weight and liver weight. Exposure to BP (up to 1200 mg/kg/day) produced no systemic toxicity based on clinical signs, body weights and liver weights (Table 3).

Table 2. Effect of BP on uterus weights in immature uterotrophic assay

Chemicals	Doses (mg/kg/day)	Absolute weights		Relative weights	
		Blotted weight (mg)	Dry weight (mg)	Blotted weight (mg/BW × 100)	Dry weight (mg/BW × 100)
Vehicle	0	29.8 ± 0.4[a]	5.3 ± 0.1	50.9 ± 0.9	8.9 ± 0.2
BP	400	59.6 ± 1.4*	11.1 ± 0.2*	106.3 ± 2.5*	19.7 ± 0.3*
	800	68.0 ± 1.0*	11.2 ± 0.2*	121.8 ± 2.1*	20.5 ± 0.4*
	1000	68.9 ± 1.4*	11.6 ± 0.3*	126.9 ± 3.2*	21.4 ± 0.5*
	1200	73.5 ± 0.8*	13.1 ± 0.2*	135.7 ± 2.2*	24.2 ± 0.6*
E_2	0.4	91.9 ± 0.6*	15.9 ± 0.1*	172.6 ± 1.8*	29.1 ± 0.3*

[a]Values are Mean ± SD (n = 10).
Immature female rats were administered butyl ρ-hydroxybenzoic acid (BP) by s.c. injection for 3 consecutive days.
* Significantly different from vehicle group ($P < 0.01$). BW, body weight; E_2, 17β-estradiol.

Table 3. Effect of BP on body weight and liver weight in castrated male rats for androgenicity

Chemicals	Doses (mg/kg/day)	Initial body weight (g)	Final body weight (g)	Liver (g)
Vehicle	0	271 ± 6.8[a]	328 ± 8.8	12.4 ± 0.8
	800	266 ± 6.5	302 ± 11.0	11.3 ± 0.7
BP	1000	268 ± 7.5	309 ± 5.5	11.4 ± 1.1
	1200	258 ± 7.5	311 ± 12.8	11.5 ± 1.1
TE	0.8	276 ± 4.8	328 ± 6.3	12.6 ± 0.6

Values are Mean ± SD (n = 10).
BP, butyl ρ-hydroxybenzoic acid; TE, testosterone esters.

Table 4. Effect of BP on sexual accessory organs/glands weights in castrated male rats for androgenicity

Doses (mg/kg/day)		Seminal vesicles (mg)	Prostate (mg)	Bulbocavernosus muscle (mg)	Glans penis (mg)	Cowper's glands (mg)
Vehicle	0	155.2 ± 42.1[a]	64.7 ± 26.2	62.9 ± 18.1	66.9 ± 8.06	5.9 ± 2.5
BP	800	78.4 ± 24.0*	50.8 ± 10.1	44.1 ± 22.4	49.3 ± 3.82	4.9 ± 0.9
	1000	79.1 ± 9.0*	40.9 ± 14.7	36.8 ± 11.6	47.9 ± 5.58	4.8 ± 1.4
	1200	87.1 ± 15.0*	52.6 ± 20.8	46.0 ± 20.5	41.4 ± 6.56	4.1 ± 1.6
TE	0.8	767.9 ± 30.8*	335.3 ± 16.4*	205.1 ± 16.3*	94.2 ± 12.3*	31.6 ± 1.5*

[a]Values are Mean ± SD (n = 10).
* Significantly different from vehicle control group ($P < 0.01$). BP, butyl ρ-hydroxybenzoic acid; TE, testosterone esters.

As shown in Table 4, TE significantly increased the weights of all sexual accessory glands and tissues examined. However, BP induced the significant decrease of the weight of the seminal vesicles with dose-dependent tendency. Other accessory organ/tissues - prostate gland, bulbocavernosus muscle, glans penis and Cowper's gland were also decreased. But these changes were not significant.

DISCUSSION

The OECD proposed that the rat uterotrophic assay and the Hershberger assay as screening methods to detect the endocrine disrupting chemicals (OECD, 2001). The uterotrophic assay was designed to assess estrogenic or antiestrogenic properties of the chemical *in vivo* (Cook *et al.*, 1997; Odum *et al.*, 1997). On the other hand, the Hershberger assay was considered to screen chemicals that have androgenic or anti-androgenic properties *in vivo* (Hershberger *et al.*, 1953; Yamada *et al.*, 2000). In this study, we performed the uterotrophic assay and the Hershberger assay to test the endocrine disrupting toxicity of BP. For validation of these assay, estradiol and testosterone derivatives (Duratestone®) were used as reference chemicals.

The ρ-hydroxybenzoic acid(parabens) was extensively used as preservatives in cosmetics, food and pharmaceutical products because of its broad spectrum of activity, worldwide legislative acceptance, biodegradability and low cost (Elder, 1984). Among parabens, BP showed high antimicrobial activity with long alkyl chain length. Accumulating data suggested that BP might have endocrine disrupting toxicity (Routledge and Sumpter, 1997; Fang *et al.*, 2001; Darbre *et al.*, 2003).

When administered alone, BP was able to give estrogen agonist response and increased the weight of blotted and dry uterus in immature rat uterotrohpic assay without any gross toxicity. This result was consistent with the previous report (Darbre *et al.*, 2003). This is interesting in view of the widespread use of parabens as preservative in the cosmetics.

In the Hershberger assay, no severe systemic toxicity was observed even at the highest dose level of BP 1200 mg/kg/day. The BP significantly reduced the weight of seminal vesicles, but the weights of other accessory organs and glands were slightly decreased.

It is concluded that BP exhibits adverse estrogenic and anti-androgenic activity and this means BP caused endocrine disrupting actions when exposed to live animals.

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