

## Hershberger Assays for Bisphenol-A and Its Substitute Candidates

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**ABSTRACT** : Bisphenol-A(BPA) is a member of alkylphenol family, and shows adverse effects including reduced fertility, reproductive tract abnormalities, metabolic disorder, cancer induction, neurotoxicity and immunotoxicity. In the present study, we conducted Hershberger assay to evaluate whether the two candidates to replace BPA have androgenic or antiandrogenic activity. The assay was carried out using immature castrated Sprague–Dawley male rats. After 7 days of the surgery, testosterone propionate (TP, 0.4 mg/kg/day) and test materials (low dose, 40 mg/kg/day; high dose, 400 mg/kg/day) were administered for 10 consecutive days by subcutaneous (s.c.) injection and oral gavage, respectively. Test materials were BPA, isosorbide (ISO) and cyclohexanedimethanol (CHDM). The rats were necropsied, and then the weights of five androgen-dependent tissues [ventral prostate, seminal vesicle, levator ani-bulbocavernosus (LABC) muscle, paired Cowper's glands, and glans penis] and three androgen-insensitive tissues (kidney, spleen and liver) were measured. All test materials including BPA did not exhibit any androgenic activity in the assay. On the contrary, antiandrogen-like activities were found in all test groups, and the order of the intensity was CHDM > BPA > ISO in the five androgen-sensitive tissues. There was no statistical difference between low dose treatment and high dose treatment of BPA group as well as ISO group. In CHDM group, high dose treatment exhibited most severe weight reduction in all measured tissues. There was no statistical difference in androgen-insensitive tissue measurements, except BPA groups. Since the effects of ISO treatment on the accessory sex organs were much less or not present at all when compared to those of BPA, ISO could be a strong candidate to replace BPA. CHDM treatment brought most severe weight reduction in all of androgen-sensitive tissues, so this material should be excluded for further screening of BPA substitute selection.

**Key words** : Rat, Hershberger assay, Bisphenol-A, Isosorbide (ISO), Substitute

### INTRODUCTION

Endocrine disrupting chemicals or compounds (EDCs) are substances that can interfere with endocrine systems in animals including human. Among them, bisphenol-A(BPA) is a member of alkylphenol family and is used primarily to make plastics, and products using BPA-based plastics have been available in commercial since 1957 (Nimrod & Ben-

son, 1996; Pivnenko et al., 2015). More recently, BPA is a preferred color developer in carbonless copy paper and thermal point of sale receipt paper (Karwacka et al., 2017). Actually, BPA seems to be most commonly exposed industrial EDC in modern society, and its adverse effects have been extensively studied over past two decades (Giulivo et al., 2016; McLachlan, 2016).

Numerous studies have demonstrated an association

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between BPA exposure and reproductive toxicities including abnormal reproductive tract formation, lower fertility, sign of early puberty and longer estrus, and abnormal breast development (Richter et al., 2007; Costa et al., 2014; Giulivo et al., 2016; Sifakis et al., 2017). BPA can mimic the structure and function of the hormone estradiol by binding to and activating the same estrogen receptors (ERs) as the natural hormone (Dickerson & Gore, 2007). Although most of BPA effects have been found in female, a considerable amount of research revealed a connection between BPA exposure and male reproductive disorders such as altered prostate cell physiology and sperm abnormalities (Ho et al., 2006; Manfo et al., 2014).

Due to many detrimental effects of BPA, particularly on the fetus and newborn (Meeker, 2012), the task making plastic products with BPA alternatives becomes much more urgent. Consequently, the potential negative effects of the candidates should be accurately and efficiently screened. So far, there are two standard evaluation procedures recommended by Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD) for initial *in vivo* screening of compounds with estrogenic or androgenic nature; Uterotrophic assay and Hershberger assay, respectively.

The Hershberger Bioassay is a short-term *in vivo* screening test using accessory tissues of the male reproductive tract to detect compounds with potential to exert androgenic or antiandrogenic activities (EDSP, 1998; OECD, 2007, 2008). This assay is relatively convenient and reliable because five androgen-dependent organs, in castrated rats, sensitively respond to chemical with androgenic or antiandrogenic activities as an increase or decrease in absolute weights. The Hershberger assay has been thought to be sensitive and specific for androgenic responses and, though it is little bit complicated, also sensitive and specific for antiandrogenic responses (Marty & O'Connor, 2014).

In the present study, we conducted Hershberger assay to

evaluate whether the two candidates to replace BPA have androgenic or antiandrogenic activity.

## MATERIALS AND METHODS

### 1. Chemicals

Testosterone propionate (TP), bisphenol-A (BPA, CAS number 80-05-7), Isosorbide (ISO, CAS number 652-67-5) and cyclohexanedimethanol (CHDM, CAS number 105-08-8) and corn oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All substances were stored in glass containers at room temperature.

### 2. Animals and ethics approval

Male Sprague-Dawley rats (28 days old) were obtained from DBL (Chungchungbuk-do, Korea) and reared in Sangmyung university animal facility under specific pathogen free (SPF)-conditions of 12-h light/dark cycle (lights on at 07:00 h) and constant temperature of  $22\pm 1^\circ\text{C}$ . Rats had free access to normal chow (Purina, Seoul, Korea) and tap water *ad libitum*. All procedures used were approved by the Animal Care and Use Committee (2017-01-03) at Sangmyung University. All the animals were handled in accordance with the guidelines for animal experiments of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

### 3. Surgery and test substance administrations

Rats were castrated (ORX) on postnatal day (PND) 42 under anesthesia by placing an incision in the scrotum and removing both testes and epididymides with a ligation of the blood vessels and seminal ducts. The animals were allowed to recover from castration for 7 days, and then animals were randomly assigned to different groups in terms of their body weights on PND 49 ( $n = 6$ ). TP was daily administered for 10 consecutive days by subcutaneous (s.c.) injection at a dose of 0.4 mg/kg bw/day (dissolved in 0.5 mL corn oil). The route of

administration for the test substances (low dose, 40 mg/5 mL/kg bw; high dose, 400 mg/5 mL/kg bw) was via oral gavage.

For the androgenic activity test, the vehicle group (ORX) was the negative control, and the TP-treated group (ORX+TP) was the positive control. The weights of the tissues were compared to the controls for statistically significant changes. Two dose groups of the test substance plus positive and vehicle (negative) controls were normally sufficient to determine if a chemical is an androgen agonist, and this design was therefore preferred for animal welfare reasons (EDSP, 1998).

#### 4. Sample collections

Approximately 24 hours after the final administration of the test substance, the rats were necropsied according to standard laboratory procedures. As critical indices, the weights of five androgen-dependent tissues [ventral prostate, seminal vesicle, coagulating glands, levator ani-bulbocavernosus (LABC) muscle, paired Cowper's glands, and glans penis] were measured. The weights of optional tissues (kidney, spleen and liver) were also measured.

#### 5. Statistical analysis

All values were expressed as mean S.E. (n=6). Absolute tissues weights were analyzed using analysis of covariance (ANOVA) or Student's *t*-test. Data were expressed as means±S.E., and *P* value<0.05 denoted the statistically significant difference.

## RESULTS

No abnormal clinical wounds or body weight changes were observed in the rats administered with the test chemicals.

#### 1. Effects on androgen-dependent tissues

The effects of BPA and replacement candidates on

changes in body weights and androgen-dependent tissues weights in castrated male rats are shown in Table 1. In all organs, the most severe weight loss was commonly found in CHDM-400+TP group.

##### 1) Body weights

Significant decreases in body weight were observed in all the BPA treatment groups except high dose group (BPA-400) when compared to their negative (ORX) and positive group (ORX+TP), respectively. However, comparison between control groups and ISO groups showed no significant changes. In CHDM groups, significant change was found only in low dose group (CHDM-40) compared to control group.

##### 2) Weights of LABC

Like body weight changes, significant decreases in LABC weight were observed in all the BPA treatment groups except high dose group (BPA-400). Co-treatment with ISO and TP groups induced significant changes, but the decrements were minimal compared to the BPA co-treated groups. In contrast, co-treatment with CHDM and TP groups exerted similar or stronger negative effects compared to BPA groups.

##### 3) Weights of glan penis

Significant decreases in glan penis weight were observed in low dose BPA treatment groups (BPA-40 and BPA-40+TP). There was no significant change in ISO groups while all CHDM group except CHDM-40+TP group showed significant decreases.

##### 4) Weights of paired Cowper's glands

Significant decreases in Cowper's gland weight were observed in BPA-40 group and BPA-400+TP group. There was no significant change in ISO groups while all CHDM group except CHDM-40 group shown significant decreases.

**Table 1. Effects of orally administered bisphenol A (BPA), Isosorbide (ISO) and cyclohexanedimethanol (CHDM) and subcutaneously administered testosterone propionate (TP) on body and androgen-sensitive organ weights of castrated rats**

Treatment <sup>a</sup>	Body weight(g)	LABC(mg)	Penis(mg)	Cowper's glands(mg)	S.V.(mg)	Prostate(mg)
INT	267.9±12.5	646.1±22.8	262.2±12.3	36.0±1.8	226.3±11.8	130.7±5.6
ORX	275.8±7.4	230.2±21.4	151.0±10.6	4.6±0.5	28.5±2.4	12.6±1.2
ORX+TP	307.3±7.3	553.2±17.5	227.5±7.7	19.0±0.8	141.2±7.7	75.0±4.9
BPA-40	240.0±5.1**	161.0±7.7*	117.3±4.4*	3.3±0.2*	22.8±1.3*	8.1±0.5**
BPA-40+TP	255.3±8.3††	459.4±13.4††	207.4±4.1†	19.1±1.1	140.9±4.1	69.0±4.0
BPA-400	254.7±6.6	209.1±9.7	132.5±5.1	3.7±0.4	27.5±1.2	11.3±0.5
BPA-400+TP	273.1±5.9††	463.3±13.5††	514.8±7.7	15.1±0.8††	131.3±5.2	71.6±2.7
ISO-40	266.3±4.0	251.6±14.0	151.1±3.6	5.9±0.7	31.1±2.8	10.2±0.8
ISO-40+TP	284.8±7.9	485.5±21.6†	212.1±3.1	17.5±1.1	127.7±12.2	51.0±1.9†††
ISO-400	281.5±9.0	238.6±11.3	151.0±6.1	5.6±0.3	25.3±2.1	11.2±0.8
ISO-400+TP	285.6±7.2	491.7±3.8††	224.2±6.6	19.2±0.5	142.0±5.4	70.9±1.7
CHDM-40	254.8±5.1*	165.9±19.2*	124.4±4.7*	3.7±0.3	19.7±1.2**	8.1±0.3**
CHDM-40+TP	293.7±6.4	450.7±16.2††	219.5±6.0	13.1±1.0†††	112.8±3.5††	56.5±3.6††
CHDM-400	274.6±8.3	170.3±14.3*	122.1±4.2*	3.5±0.2*	17.4±1.0***	9.2±0.7*
CHDM-400+TP	290.9±6.6	398.6±21.5†††	187.8±8.9††	10.8±0.9†††	82.5±5.3†††	36.5±3.7†††

<sup>a</sup> Castrated immature rats were administered with TP by subcutaneous injection for 10 days. One day after the final treatment, the accessory sex organs were removed carefully and weighed separately.

Symbols \*, \*\* and \*\*\* meant significant difference in comparison between ORX group and test material group. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ .

Symbols †, †† and ††† meant significant difference in comparison between ORX group and test material group. †,  $P<0.05$ ; ††,  $P<0.01$ ; †††,  $P<0.001$ .

### 5) Weights of seminal vesicle

No significant change was observed in BPA groups except BPA-40 group. There was no significant change in ISO groups while all CHDM group showed significant decreases.

### 6) Weights of ventral prostate

No significant change in ventral prostate weight was observed in all the BPA and ISO groups except BPA-40 and ISO-40+TP groups. However, the weights of all the

CHDM groups were significantly decreased.

## 2. Effects on androgen-independent organs

The effects of BPA and replacement candidates on changes in optional organs (liver, kidney and spleen) weights in castrated male rats are shown in Tables 2.

### 1) Weights of kidney

Significant decreases in kidney weight were observed in all BPA treatment groups when compared to their negative

**Table 2. Effects of orally administered bisphenol A (BPA), isosorbide (ISO) and cyclohexanedimethanol (CHDM) and subcutaneously administered testosterone propionate (TP) on androgen-insensitive organ weights of castrated rats**

Treatment <sup>a</sup>	Kidney(mg)	Spleen(mg)	Liver(g)
INT	1,117.8±17.1	696.1±33.7	11.0±0.4
ORX	1,063.9±16.1	688.1±34.1	11.3±0.5
ORX+TP	1,098.3±18.1	895.4±55.8	12.2±0.4
BPA-40	879.7±9.5 <sup>***</sup>	634.0±25.6	9.2±0.3 <sup>**</sup>
BPA-40+TP	1,006.4±15.6 <sup>†††</sup>	746.6±59.1	11.0±5.2
BPA-400	935.6±25.3 <sup>***</sup>	625.3±43.2	10.1±0.4
BPA-400+TP	1,044.2±17.7 <sup>†</sup>	629.0±20.7 <sup>††</sup>	10.9±0.3 <sup>†</sup>
ISO-40	1,045.5±18.9	803.9±71.6	10.2±0.2
ISO-40+TP	1,092.7±22.7	720.5±21.1 <sup>†</sup>	10.8±0.4 <sup>†</sup>
ISO-400	1,038.1±26.7	744.6±31.5	11.2±0.5
ISO-400+TP	1,111.4±29.5	794.2±48.3	11.8±0.4
CHDM-40	965.6±21.2 <sup>**</sup>	666.8±28.0	10.1±0.3
CHDM-40+TP	1,117.7±14.5	761.6±38.2	12.3±0.3
CHDM-400	1,036.0±38.7	706.2±33.7	11.0±0.4
CHDM-400+TP	1,164.0±20.9 <sup>†</sup>	789.6±47.9	12.4±0.4

<sup>a</sup> Castrated immature rats were administered with TP by subcutaneous injection for 10 days. One day after the final treatment, the accessory sex organs were removed carefully and weighed separately.

Symbols \*, \*\* and \*\*\* meant significant difference in comparison between ORX group and test material group. \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$ .

Symbols †, †† and ††† meant significant difference in comparison between ORX group and test material group. †,  $P<0.05$ ; ††,  $P<0.01$ ; †††,  $P<0.001$ .

control and positive control, respectively. However, comparison between control groups and ISO groups exerted no significant changes. In CHDM groups, significant change was found in CHDM-40 group and CHDM-400+TP group compared to control group.

## 2) Weights of spleen

Unlike kidney weight change, no significant change in spleen weight was observed in all the BPA group except

BPA-400+TP group. Also, there was no significant weight change in all ISO group and CHDM group except ISO-40+TP group.

## 3) Weights of liver

Significant decreases in liver weight were observed in BPA-40 group and BPA-400+TP group. As spleen weights, there was no significant weight change in all ISO group and CHDM group except ISO-40+TP group.

## DISCUSSION

The extended definition of EDC is a structurally diverse class of synthetic and natural compounds that possess the ability to alter various component of the endocrine system and potentially induce adverse effects in exposed individuals and populations (Devillers et al., 2006). Indeed, EDCs encompass wide range of synthetic and natural compounds, and the list is getting longer. Simultaneously, a body of evidence on the adverse effects of EDCs is getting larger, so EDCs-related health issues have become a matter of global concern (WHO, 2013). For example, BPA is one of the highest volume chemicals produced worldwide since 1957, and this compound is frequently used for food and beverage storage containers and thermal paper (Rubin, 2011). Like other alkylphenol family members (e.g. octylphenol and nonylphenol), BPA shows xenoestrogen exhibiting estrogen-mimicking properties (Nimrod & Benson, 1996). Numerous studies have demonstrated adverse health effects attributed to BPA including a range of reproductive problems such as reduced fertility, male and female reproductive tract abnormalities, metabolic disorder, cancer induction, neurotoxicity and immunotoxicity (Richter et al., 2007; Manfo et al., 2014; Karwacka et al., 2017; Giulivo et al., 2016; Ho et al., 2006; Itoh et al., 2012; Kimber, 2017).

Due to these BPA associated negative health effects, structural analogues, such as bisphenol S (BPS) have been introduced into the market and many substitutes are planned to be launched. However, little is known about the presence and toxicity of these emerging replacements, and concern has risen about them (Björnsdotter et al., 2017). Indeed, recent studies clearly demonstrated that two such BPA substitutes, BPS and bisphenol F (BPF), are hormonally active as BPA and have similar endocrine-disrupting effects (Eladak et al., 2015; Rochester & Bolden, 2015). In this context, development of standard evaluation procedure(s) being able to screen the substitute candidates with

good accuracy and efficiency is high priority need.

Recent studies on the BPA actions revealed that interpretation of the Hershberger or uterotrophic assay results could be tangled job. For example, emerging data show that BPA binds with a significant number of hormone receptors, including a number of nuclear and membrane-bound estrogen receptors, androgen receptors, as well as the thyroid hormone receptor, glucocorticoid receptor, and peroxisome proliferator-activated receptor gamma (PPAR  $\gamma$ ) (Teng et al., 2013; Yoon et al., 2014; MacKay & Abizaid, 2017). In particular, MacKay & Abizaid (2017) postulated that this increased diversity of receptor targets may allow to explain the puzzling aspects of BPA and other EDCs pharmacology. The weight reduction effects by antiandrogen-like activities of BPA and test materials in the present study could not be originated from a single receptor activation but from multiple ones.

In the present study, we found that all test materials including BPA did not exhibit any androgenic activity in the Hershberger assay. The lack of androgenicity and weight-loss effects of BPA in the present study are in good agreement with previous reports (Kim et al., 2002; Nishino et al., 2006). Rather, antiandrogen-like activities were found in all test materials, and the order of the intensity was CHDM > BPA > ISO in 5 androgen-sensitive organs. There is no available information on the action mechanism of ISO and CHDM (i.e. their receptors), so we tentatively concluded the weight reduction effects of these materials are antiandrogen-like activities. Meanwhile, no androgenic or antiandrogenic activities of the test materials were found in the androgen-insensitive organs used in this study. In general, the effects of ISO treatment on the accessory sex organs were much less or not present at all when compared to those of BPA. Therefore, at this level of screening, ISO could be a BPA substitute. CHDM treatment brought most severe weight reduction in all of androgen-sensitive organs, so this material should be excluded for

further screening of BPA substitute selection.

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