Original Article

Comparison of the developmental/reproductive toxicity and hepatotoxicity of phthalate esters in rats using an open toxicity data source

Kaori Ambe, Yuko Sakakibara, Aya Sakabe, Hayato Makino, Tatsuya Ochibe
and Masahiro Tohkin

Department of Regulatory Science, Graduate School of Pharmaceutical Sciences, Nagoya City University,
3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

(Received September 28, 2018; Accepted January 9, 2019)

ABSTRACT — Phthalate esters (PEs) are widely used as plasticizers in various kinds of plastic products. Some PEs have been known to induce developmental and reproductive toxicity (DART) as well as hepatotoxicity in laboratory animals. In some cases of DART, the strength of toxicity of PEs depends on the side chain lengths, while the relationship between hepatotoxicity and side chain length is unknown. Therefore, in this study, we compared DART and hepatotoxicity in rats, focusing on 6 PEs with different side chains. We collected toxicity data of 6 PEs, namely, *n*-butyl benzyl phthalate (BBP), di-*n*-butyl phthalate (DBP), di(2-ethylhexyl) phthalate (DEHP), di-isodecyl phthalate (DIDP), di-isononyl phthalate (DINP), and di-*n*-octyl phthalate (DNOP), from open data source, then, we constructed the toxicity database to comprehensively and efficiently compare the toxicity effects. When we compared DART using the toxicity database, we found that BBP, DBP, and DEHP with short side chains showed strong toxicities against the reproductive organs of male offspring, and the No-Observed-Adverse-Effect Levels (NOAELs) of BBP, DBP, and DEHP were lower than DIDP, DINP, and DNOP with long side chains. Comparing hepatotoxicities, the lowest NOAEL was shown 14 mg/kg/day for DEHP, based on liver weight gain with histopathological changes. However, as BBP and DBP showed higher NOAEL than the other 3 PEs (DIDP, DINP, and DNOP), we conclude that hepatotoxicity does not depend on the length of side chain. Concerning side chain length of PEs, we effectively utilized our constructed database and found that DART and hepatotoxicity in rats showed different modes of toxicities.

Key words: Developmental and reproductive toxicity, Hepatotoxicity, Phthalate esters

INTRODUCTION

Phthalate esters (PEs) are commonly used as a plasticizer in polyvinyl chloride (PVC) products. It is possible to exude and migrate PEs from PVC products because PEs are physically dispersed in PVC. Since PVC is used in materials such as food packaging materials, medical instruments, and children's toys, humans are potentially exposed to PEs. In laboratory animals, some PEs have been known to cause developmental and reproductive toxicity (DART) and hepatotoxicity. Regarding DART, the influence of PEs on sexual differentiation of male offspring has been reported in rats (Mylchreest *et al.*, 1998; Parks *et al.*, 2000; Wolf *et al.*, 1999). In addition, negative effects on reproductive ability and skeleton formation have been confirmed (Ema *et al.*, 1992, 1993;

Koizumi *et al.*, 2000). Therefore, DART of PEs is drawing attention based on their possible influence on future generations. On the other hand, hepatotoxicity is commonly observed as systemic toxicity in rodents that are exposed to PEs. It has been reported that some PEs cause peroxisome proliferation and liver tumors via peroxisome proliferator activated receptor alpha (PPAR α) (Ward *et al.*, 1998). Since there are species differences in PPAR α , it has been proposed that PPAR α induction and peroxisome proliferation do not occur in humans (IARC, 2000). However, in recent years, it has been shown that PPAR α activation and peroxisome proliferation are not essential for liver tumorigenesis (Guyton *et al.*, 2009; Ito *et al.*, 2007). Furthermore, some PEs have lower "No-Observed-Adverse-Effect Level" (NOAEL) toward hepatotoxicity than DART (Fabjan *et al.*, 2006). Therefore, hepatotoxicity is

considered a serious concern for PE-induced toxicity in addition to DART.

In general, PEs are substances with two carboxyl groups in the *ortho* position; there are those in which a linear alkyl group, a branched alkyl group, or a benzyl group is ester-bonded. In some cases of DART, the toxicity of PEs varies widely, depending on their side chains (Gray *et al.*, 2000; Koizumi *et al.*, 2001). Fabjan *et al.* (2006) concluded that PEs whose side chain length range from C4 to C6 induced similar severe reproductive effects in animals used for category approach analysis. However, there has been little research on the relationship between hepatotoxicity and the side chain lengths of PEs. Consequently, it is important to clarify the toxicities of PEs with similar structures by comparing their DART and the hepatotoxicity comprehensively, and analyzing the relationships between these toxicities and the lengths of the side chain.

In this study, we collected public information about the DART and the hepatotoxicity of 6 PEs with different side chains from an open toxicity data source. *N*-butyl benzyl phthalate (BBP), di-*n*-butyl phthalate (DBP), and di(2-ethylhexyl) phthalate (DEHP) are PEs with short side chains (from C4 to C6); while di-isodecyl phthalate (DIDP), di-isononyl phthalate (DINP), and di-*n*-octyl phthalate (DNOP) are PEs with long side chains (from C8 to C9) (Fig. 1). These 6 PEs consist of the phthalate basic structure and different side chain lengths, and they are all substances subject to regulation under the Food Sanitation Act (<https://www.mhlw.go.jp/topics/bukyoku/iyaku/kigu/dl/100906-1.pdf>, last accessed September 1, 2018). We exhaustively investigated the DART on traits such as reproductive function of the parent generation and development of the next generation, and then compared the toxicities among the 6 PEs. To comprehensively investigate toxicity, an effective measure is to organize the database because DART is extremely complicated and diverse. In addition, such a database is considered useful in the category approach to predict the toxicity of similarly structured substances (NAFTA, 2012; OECD, 2014). Therefore, we first constructed a database of the DART and organized the hepatotoxicity information. We then analyzed the toxicological effects of 6 PEs.

MATERIALS AND METHODS

Data collection

Information on the DART and the hepatotoxicity of 6 PEs (BBP, DBP, DEHP, DIDP, DINP, DNOP) were collected from the apparatus and containers/packages risk assessment reports of the Food Safety Commission of

Fig. 1. Chemical structure formula of 6 phthalate esters (PEs). Carbon numbers (C4-C9) involved in the linear side chains of each PE are indicated.

Japan (http://www.fsc.go.jp/english/evaluationreports/apparatcontainers_e3.html, last accessed June 1, 2018). As the risk assessment of PEs utilized high quality existing information, the risk assessment reports contain literature information and public information of regulatory authorities, in which the quality of data is secured in the risk assessment. We extracted the results of oral administration tests on all animals, specifically, the generation reproduction toxicity tests, developmental toxicity tests, repeated dose toxicity tests, and carcinogenicity tests.

Extraction of toxicity data

We extracted the chemical substance information and test condition information of each test. Chemical substance information consisted of CAS number and chemical substance name. Test condition information consisted of type of test, animal species, strain, exposure period, route of administration, Lowest-Observed-Adverse-Effect Level (LOAEL), NOAEL, literature information, and reliability of literature. These items of information were entered and organized on a Microsoft Excel worksheet (Microsoft Excel 2013, Microsoft Corporation, Redmond,

Comparison of toxicity effects of phthalate esters

WA, US), which we called a test datasheet. If there were multiple pieces of toxicity test information for one PE, all the test information were entered.

A matrix table of dose and toxicity findings was created on the test datasheet for each test. Toxicity findings were summarized by several categories according to OECD guidelines (OECD, 2008). Similar toxicity findings were classified under the same category. Major items of categories are shown in supplementary Table 1. If a corresponding toxicity finding was observed, a value of “1” was entered in each cell of the table. On the other hand, if there were no toxicity findings, the cells were left blank. Detailed descriptions such as general clinical observations, gross necropsy findings, and histopathologic findings were written as descriptive expressions.

In the developmental toxicity test, the endpoint varied depending on the administration period. Therefore, in this study, each test was divided into 6 administration periods (I - VI) based on the stage of developmental/reproductive processes (ICH, 2005). Each administration period was defined as follows: Period I is from premating to conception; Period II is from conception to implantation; Period III is from implantation to closure of the hard palate; Period IV is from closure of the hard palate to the end of pregnancy; Period V is from birth to weaning; Period VI is from weaning to sexual maturity. It is assumed that implantation in rats occurs on days 6-7 of pregnancy, and closure of the hard palate occurs on days 15-18 of pregnancy.

The reliability of the literature was evaluated using the Klimisch code consisting of 4 steps (Klimisch *et al.*, 1997). Test results based on official guidelines, for example Good Laboratory Practice, is assigned a value of “1”, which is the most reliable code. Data not fully compliant or not stated in the guidelines but sufficient to be acceptable are assigned a value of “2”. Code 3 corresponds to unreliable data and code 4 corresponds to unevaluable data.

DART information was managed using FileMaker Pro 16 (FileMaker, Inc. Santa Clara, CA, US), which is

a relational database software; we constructed a unified information management and retrieval system by linking the test datasheets for each PE. This database was also linked to literature, risk assessment reports, and Simplified Molecular Input Line Entry System (SMILES). SMILES are chemical structure information obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>, last accessed April 1, 2018).

Comparison of toxicity effects

We evaluated the DART effects on male and female reproductive organs, reproductive ability, and teratogenicity. In this study, teratogenicity was defined as structural alterations in the development, accordingly, malformation and variation on external, visceral, and skeletal examination was evaluated. The definition of malformation is a permanent structural change that has a detrimental effect, and variation is a structural change with little effect on animals (OECD, 2018). However, structural alterations in the reproductive organs were summarized as effects on the reproductive organs. For each of these detailed endpoints, the lowest LOAEL and NOAEL for each PE was compared using our database. To evaluate hepatotoxicity, the lowest LOAEL and NOAEL of effects on liver were extracted by using our DART database in addition to repeated dose toxicity tests and carcinogenicity tests. We compared toxicity effects only in rats because toxicity data of all 6 PEs were available only in rats.

RESULTS AND DISCUSSION

Collected tests of DART and hepatotoxicity

Although we collected the DART and hepatotoxicity information of rat, mouse, rabbit, dog, and marmoset from the risk assessment reports of the Food Safety Commission of Japan, we analyzed the toxicity data of rats because the necessary toxicity data of all 6 PEs was available only in rats. We collected DART information from a total of 62 oral administration tests on rats (Table 1). The number of tests collected for each PE were 14 tests

Table 1. Number of collected tests on rat.

Test type	BBP	DBP	DEHP	DIDP	DINP	DNOP	Total
One-generation test	1	0	0	1	1	0	3
Two- or three-generation test	3	2	3	2	1	0	11
Developmental toxicity test	10	21	12	2	2	1	48
Sub total	14	23	15	5	4	1	62
Repeated dose toxicity test	8	3	9	5	6	1	32
Carcinogenicity test	3	1	3	2	2	0	11
Sub total	11	4	12	7	8	1	43
Total	25	27	27	12	12	2	105

for BBP, 23 tests for DBP, 15 tests for DEHP, 5 tests for DIDP, 4 tests for DINP, and 1 test for DNOP. In terms of test type, information for 3 one-generation reproduction toxicity tests, 11 two or three-generation reproduction toxicity tests, and 48 developmental toxicity tests were collected. The number of tests for BBP, DBP, and DEHP, especially in the developmental toxicity test, were larger than those for DIDP, DINP, and DNOP. Since DART has been noted in BBP, DBP, and DEHP, we consider the number of tests to be large. The information for DNOP was limited because there was only one test of developmental toxicity conducted on rats. In the developmental toxicity test, we separated each test into 6 categories according to the administration period (I - VI); then, we counted the number of tests included in each period (Table 2). The number of tests from period III to IV were the largest, and there was a total of 14 tests. The next most frequent test was for the III period only, and there was a total of 13 tests. The III period covers the period from implantation to closure of the hard palate, and the IV period covers the period from closure of the hard palate to the end of pregnancy. Although there were many developmental toxicity tests for BBP, DBP, and DEHP, the administration period was duplicated. In this study, we collected toxicity data that included the III and IV periods for all 6 PEs; thus, we conducted a comprehensive investigation on developmental toxicity especially teratogenicity.

We collected the toxicity data from the repeated dose toxicity test and the carcinogenicity test to investigate hepatotoxicity. The information from 43 tests for hepatotoxicity were collected (Table 1). These tests consisted of 11 tests for BBP, 4 tests for DBP, 12 tests for DEHP, 7 tests for DIDP, 8 tests for DINP, and 1 test for DNOP. Although only one test was obtained for DNOP, it was possible to compare the hepatotoxicity using general toxicity test information of all 6 PEs. The number of collected tests of DART and hepatotoxicity by animal species (mouse, rabbit, dog, and marmoset) other than rat were listed in supplementary Table 2.

Comparison of DART among 6 PEs

Using our DART database and focusing on rat oral administration tests, we compared the lowest LOAEL and NOAEL of each PE to analyze the toxicity effects on male and female reproductive organs, reproductive ability, and teratogenicity. All tests used for comparison were evaluated as either Klimisch code 1 or 2, thus the information were considered reliable. In this study, we adopted LOAEL and NOAEL of reproductive and developmental toxicity in the generation reproduction toxicity test used for comparing DART.

Regarding PE effects on male reproductive organs, BBP, DBP, and DEHP showed strong effects on offspring, including the delay of formation of spermatocytes and reduction of anogenital distance (AGD) (Table 3).

Table 2. Number of the developmental toxicity tests for periods I-VI.

Period	BBP	DBP	DEHP	DIDP	DINP	DNOP	Total
I	0	0	0	0	0	0	0
II	0	2	0	0	0	0	2
III	5	4	1	2	1	0	13
IV	0	0	0	0	0	0	0
V	0	0	0	0	0	0	0
VI	0	0	0	0	0	0	0
I-IV	1	0	0	0	0	0	1
I-V	4	0	0	0	0	0	4
II-III	0	1	0	0	0	0	1
II-IV	0	0	2	0	0	0	2
II-V	0	2	1	0	0	0	3
II-VI	0	1	0	0	0	0	1
III-IV	0	10	3	0	0	1	14
III-V	0	0	3	0	0	0	3
IV-V	0	1	0	0	1	0	2
Others	0	0	2	0	0	0	2
Total	10	21	12	2	2	1	48

Period I is from pre mating to conception; Period II is from conception to implantation;

Period III is from implantation to closure of the hard palate; Period IV is from closure of the hard palate to the end of pregnancy;

Period V is from birth to weaning; Period VI is from weaning to sexual maturity.

Comparison of toxicity effects of phthalate esters

DEHP, which has a C6 side chain with an ethyl group at the 2nd position (Fig. 1), showed the lowest NOAEL (3 mg/kg/day) among the 6 PEs, based on reduced the AGD and decreased the weight of the reproductive organ of offspring. The NOAEL of PEs with the longer side chain (DIDP, DINP, DNOP) was 100 mg/kg/day or more, thus the NOAEL of DEHP was between one-thirtieth and one-three hundredth of the NOAEL of DIDP, DINP, and DNOP. The same trend was observed for the LOAELs (Table 3). Since the NOAEL was not obtained for BBP and DBP, the LOAELs were compared. DBP has a linear C4 side chain, and BBP has one of the butyl groups of DBP replaced by a benzyl group (Fig. 1). DBP, with the lowest LOAEL (1.5-3.0 mg/kg/day) among the 6 PEs, delayed the formation of spermatocytes in offspring. The LOAEL of DBP was between one-two hundredth and one-millionth of the LOAELs of DIDP and DINP. BBP showed testicular toxicity in parent animals and offspring with a LOAEL of 100 mg/kg/day. The LOAEL of BBP was between one-half and one-tenth of the LOAELs of DIDP and DINP. DNOP had no effect on male reproductive organs even at the highest dose of 1000 mg/kg/day. Furthermore, because the results BBP, DIDP, and DINP were obtained from the generation reproduction toxicity tests, the administration periods of BBP, DIDP, and DINP were longer than those of DBP and DEHP. However, the LOAELs of DBP and DEHP were lower than those of BBP, DIDP, and DINP. These results indicate that DBP and DEHP showed the strongest toxicity toward male reproductive organs among the 6 PEs.

The administration of chemical substances mater-

nally from late pregnancy to early lactation is known to influence the reproductive organs on offspring (Wine *et al.*, 1997; Wolf *et al.*, 1999). These processes correspond to period IV and V in this study. The tests for BBP and DINP included the developmental toxicity tests for IV and V periods (Table 2); the generation reproduction toxicity test showed lower LOAEL than the developmental toxicity test (Table 3). The tests for DIDP included only two developmental toxicity tests with the III period (from implantation to closure of the hard palate); therefore, this PE was evaluated using the 2-generation test (Table 2). In addition, the effects of DIDP and DINP on the male parent reproductive organs were stronger than on the offspring. Although DIDP with a range of LOAELs of 211-405 mg/kg/day delayed the formation of spermatocytes in parents, no effect on male reproductive organs of offspring were found even at the highest dose of 1000 mg/kg/day or more (Hushka *et al.*, 2001). In the development toxicity test not listed in Table 3, DINP reduced the testicular weight of offspring at the LOAEL of 1,165-2,657 mg/kg/day during the administration period of Gestation Day (GD) 15 – Postnatal Day (PND) 10 (Masutomi *et al.*, 2003). These results indicate that DIDP and DINP had little influence on the male reproductive organs of offspring.

To summarize the PE effects on male reproductive organs, BBP, DBP, and DEHP with short side chains clearly influenced the reproductive organs of offspring, while DIDP, DINP, and DNOP with long side chains had little influence on offspring. Therefore, these results confirm the previous reports that PEs with short side chains

Table 3. Effects of the phthalate esters on male reproductive organs.

PEs	Literature information	Animal	Exposure ^{a)}	LOAEL (mg/kg/day)	NOAEL (mg/kg/day)	Major toxic effect
BBP	Aso <i>et al.</i> , 2005	CD(SD) IGS rat	2 generations, gavage	100	None ^{b)}	Parents: testicular softening and sperm reduction, Offspring: reduction of AGD
DBP	Lee <i>et al.</i> , 2004	CD(SD) IGS rat	GD 15 - PND 21, diet	1.5-3.0	None ^{b)}	Offspring: formation delay of spermatocytes
DEHP	Christiansen <i>et al.</i> , 2010	Wistar rat	GD 7 - PND 16, gavage	10	3	Offspring: decreased reproductive organ weight and reduction of AGD
DIDP	Hushka <i>et al.</i> , 2001	SD rat	2 generations, diet	211-405	103-198	Parents: increased epididymal absolute weight
DINP	Waterman <i>et al.</i> , 2000	SD rat	1 generation, diet	966-1,676	622-1,157	Parents: increased testicular absolute weight
DNOP	Saillenfait <i>et al.</i> , 2011	SD rat	GD 6 - 20, gavage	None ^{c)}	1000	Even at the highest dose, no effect was seen.

a) duration, route

b) NOAEL could not be set because of the lowest dose.

c) LOAEL could not be set because of the highest dose.

AGD, anogenital distance; GD, gestation day; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level; PND, postnatal day

ranging from C4 to C6 elicit severe testicular toxicity (Fabjan *et al.*, 2006). However, BBP was weakly toxic compared to the extremely strong toxicity of DBP and DEHP. Regarding the mechanism of testicular toxicity, androgen is a male steroid hormone synthesized in the testis; therefore, the PE effects on male reproductive organs, such as shortened AGD and testicular malfunction, may be anti-androgenic actions. It is also known that chemical substances suspected anti-androgenic effects cause shortened AGD and increased nipple retention, especially in male offspring (Gray *et al.*, 2001; McIntyre *et al.*, 2001). DEHP increased the incidence of nipple retention at the 10 mg/kg/day which was the same dose as AGD shortening (Christiansen *et al.*, 2010). Although the target molecule is unknown, the testicular toxicity of DBP and DEHP is thought to be the result of an indirect anti-androgenic action not through the androgen receptor (Mylchreest *et al.*, 1999; Wolf *et al.*, 1999). A more detailed examination into the relationship between side chain structure and anti-androgenic actions is required.

Analysis of the effects of 6 PEs on female reproductive organs showed that BBP, with the lowest NOAEL (100 mg/kg/day) among the 6 PEs, elicited weight loss of the ovary in parent animals (Table 4). The NOAEL of BBP ranged between half to one-tenth of other PEs (DBP, DEHP, DIDP, DINP, DNOP). DNOP had no effect on female reproductive organs even at the highest dose of 1000 mg/kg/day. All these effects on female reproductive organs were observed in parent animals. Regarding the administration period, since the NOAELs of BBP, DIDP, and DINP were obtained from 2-generation tests, and the NOAELs of DBP and DEHP were obtained from developmental toxicity tests, the observation periods of BBP, DIDP, and DINP were longer than those of DBP and DEHP. In the 2-generation test not listed in Table 4, DBP had no effect on female reproductive organs even at the highest dose of 794 mg/kg/day (Wine *et al.*, 1997). DEHP had no effect on female reproductive organs even at the highest dose of 775 mg/kg/day in the 3-generation test (Wolfe and Layton, 2004). These results indicate that the effects of PEs on female reproductive organs are weak. The range of values of the LOAELs and NOAELs of 6 PEs was narrow, and was not related to the length of the side chain; thus, it appears that the female and male reproductive organs are affected differently. Regarding the estrogenic actions, it has been reported that BBP, DBP, and DEHP do not cause uterine hypertrophy on rats (Coldham *et al.*, 1997; Zacharewski *et al.*, 1998). Therefore, it is likely that PEs have little estrogenic activity, and their effects on female reproductive organs are weak.

Reproductive toxicity effects were clearly observed for

BBP, DBP, and DEHP with short side chains (Table 5). Among the 6 PEs, the lowest NOAEL was 46 mg/kg/day for DEHP, based on the decrease of pregnancy index. The NOAEL of DEHP was one-twentieth that of DIDP and DINP. On the other hand, BBP showed NOAEL of 250 mg/kg/day, based on the decrease of mating and fertility indexes. The NOAEL of BBP was one-quarter that of DIDP and DINP. In contrast, DIDP and DINP with long side chains had no effect on reproductive ability even at the highest dose. We were unable to find generation test information for DNOP, thus we could not evaluate the reproductive effects of DNOP. Comparing the LOAELs, DBP, with the lowest LOAEL (52-80 mg/kg/day) among the 6 PEs, decreased the number of surviving offspring. To summarize, BBP, DBP, and DEHP, which have side chains ranging from C4 to C6, affected reproductive ability. Reproductive toxicity followed the same tendency as that found in male reproductive organs, i.e., it was related to side chain length. However, when comparing the LOAELs of BBP, DBP, and DEHP, the reproductive toxicity is much higher than the effects on the male reproductive organs. Although the mechanism of reproductive toxicity is unknown, it may involve effects on male reproductive organs.

For developmental toxicity, we focused on teratogenicity. DIDP showed the lowest NOAEL (100 mg/kg/day) among the 6 PEs, based on skeletal variations in the fetus (Table 6). Comparing the LOAELs, DNOP, with the lowest LOAEL of 250 mg/kg/day among the 6 PEs, elicited skeletal variations in the fetus. The LOAEL of DNOP ranged between half and one-quarter that of other PEs. All the adopted tests included the III period (from implantation to closure of the hard palate). Teratogenicity other than reproductive organs is often observed after maternal administration of chemical substances at GD 6-15, which is the organogenesis period in rats. Therefore, we could compare the teratogenic effect of the 6 PEs from data listed in Table 6. Although the mechanism concerning teratogenicity has not been sufficiently clarified, it is likely not to depend on the length of the side chain. Teratogenic effects are considered to be distinct from the effects on male reproductive organs and reproductive ability.

To summarize the results of DART of 6 PEs, DEHP showed the lowest NOAEL (3 mg/kg/day) among the 6 PEs, based on the most toxic effects on male reproductive organ of offspring (Table 3). Moreover, tests for both male reproductive organs of offspring and reproductive toxicity were highly sensitive to DBP, DEHP, and BBP with short side chains of C4 to C6, although the toxic effects on male reproductive organs were observed at lower doses than those eliciting reproductive toxicity

Comparison of toxicity effects of phthalate esters

Table 4. Effects of the phthalate esters on female reproductive organs.

PEs	Literature information	Animal	Exposure ^{a)}	LOAEL (mg/kg/day)	NOAEL (mg/kg/day)	Major toxic effect
BBP	Nagao <i>et al.</i> , 2000	CD(SD)IGS rat	2 generations, gavage	500	100	Parents: decreased ovary weight
DBP	Mylchreest <i>et al.</i> , 1998	CD(SD) rat	GD 3 - PND 20, gavage	500	250	Parents: decreased uterus weight
DEHP	Hellwig <i>et al.</i> , 1997	Wistar rat	GD 6 - 15, gavage	1000	200	Parents: decreased uterus weight
DIDP	Hushka <i>et al.</i> , 2001	SD rat	2 generations, diet	508-1,582	253-761	Parents: decreased ovary and uterus weight
DINP	Waterman <i>et al.</i> , 2000	SD rat	2 generations, diet	555-1,129	287-553	Parents: decreased ovary weight
DNOP	Saillenfait <i>et al.</i> , 2011	SD rat	GD 6 - 20, gavage	None ^{b)}	1000	Even at the highest dose, no effect was seen

a) duration, route

b) LOAEL could not be set because of the highest dose.

GD, gestation day; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level; PND, postnatal day

Table 5. Reproductive toxicity of the phthalate esters.

PEs	Literature information	Animal	Exposure ^{a)}	LOAEL (mg/kg/day)	NOAEL (mg/kg/day)	Major toxic effect
BBP	Tyl <i>et al.</i> , 2004	CD(SD) rat	2 generations, diet	750	250	Decreased mating index, fertility index, number of implantations, and number of surviving offspring
DBP	Wine <i>et al.</i> , 1997	SD rat	2 generations, diet	52-80	None ^{b)}	Decreased number of surviving offspring
DEHP	Wolfe and Layton, 2004	SD rat	3 generations, diet	359	46	Decreased pregnancy index
DIDP	Hushka <i>et al.</i> , 2001	SD rat	1 generation, diet	None ^{c)}	1000	Even at the highest dose, no effect was seen
DINP	Waterman <i>et al.</i> , 2000	SD rat	1 generation, diet	None ^{c)}	1000	Even at the highest dose, no effect was seen
DNOP ^{d)}	—	—	—	—	—	—

a) duration, route

b) NOAEL could not be set because of the lowest dose.

c) LOAEL could not be set because of the highest dose.

d) DNOP had no data on reproductive performance.

LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level

(Table 3 and 5). On the other hand, the toxic effects on female reproductive organs and teratogenicity were weak compared to the effects on the male reproductive organs, and they were not related to the length of the side chain (Table 4 and 6). From these results indicate that toxicity and strong susceptibility vary for each PE. Since DART is complex and diverse, individual endpoints need to be carefully considered. In addition, PEs are considered to have biological effects as monoesters in which one ester has been hydrolyzed (Lake *et al.*, 1977). As for DBP, test data for monobutyl phthalate (MBP), which is the precursor

of DBP, showed the same tendency as DBP, indicating that DBP may elicit its toxic effects via its metabolite MBP. However, we were not able to clarify the toxicity of metabolites of 6 PEs because we were unable to obtain test data on all such metabolites.

Comparison of hepatotoxicity among 6 PEs

From our database of the DART and the general toxicity tests, we extracted the lowest values of LOAEL and NOAEL of liver effects in rats (Table 7). All tests used in the comparison were Klimisch coded 1 or 2, mean-

Table 6. Teratogenicity of the phthalate esters.

PEs	Literature information	Animal	Exposure ^{a)}	LOAEL (mg/kg/day)	NOAEL (mg/kg/day)	Major toxic effect
BBP	Ema <i>et al.</i> , 1992	Wistar rat	GD 7 - 15, gavage	750	500	Skeletal and external malformations
DBP	Ema <i>et al.</i> , 1993	Wistar rat	GD 7 - 15, gavage	630	500	External malformations
DEHP	Hellwig <i>et al.</i> , 1997	Wistar rat	GD 6 - 15, gavage	1000	200	Skeletal variations and malformations
DIDP	Waterman <i>et al.</i> , 1999	SD rat	GD 6 - 15, gavage	500	100	Skeletal variations
DINP	Hellwig <i>et al.</i> , 1997	Wistar rat	GD 6 - 15, gavage	1000	200	Skeletal variations
DNOP	Saillenfait <i>et al.</i> , 2011	SD rat	GD 6 - 20, gavage	250	None ^{b)}	Skeletal variations

a) duration, route

b) NOAEL could not be set because of the lowest dose.

GD, gestation day; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level

Table 7. Hepatotoxicity of the phthalate esters.

PEs	Literature information	Animal	Exposure ^{a)}	LOAEL (mg/kg/day)	NOAEL (mg/kg/day)	Major toxic effect
BBP	Aso <i>et al.</i> , 2005	CD(SD)IGS rat	2 generations, gavage	200	100	Parents: increased liver weight of male and female
	NTP, 1997	F344/N rat	26 weeks, diet	550	180	Adults: increased liver weight of male
DBP	Marsman, 1995	F344/N rat	GD 0 - PND 28 and 4 weeks, diet	275	133	Offspring: increased liver weight of female
	NTP, 2003	Wistar	3 months, diet	688-816	142-162	Adults: decreased fat deposition of hepatocytes of male and female
DEHP	Wolfe and Layton, 2004	SD rat	3 generations, diet	46	14	Parents: increased liver weight and histopathological changes of male and female
	Mitchell <i>et al.</i> , 1985	Wistar	9 months, diet	50	None ^{b)}	Adults: increased liver weight and peroxisome proliferation of male and female
DIDP	Hushka <i>et al.</i> , 2001	SD rat	2 generations, diet	201	60	Parents: increased liver weight of female
	EU RAR, 2003	SD rat	90 days, diet	120	60	Adults: increased liver weight of female
DINP	Waterman <i>et al.</i> , 2000	SD rat	2 generations, diet	143-285	None ^{b)}	Parents: histopathological changes in the liver of male and female
	Lington <i>et al.</i> , 1997	F344/N rat	2 years, diet	152-184	15-18	Adults: increased liver weight of male and female
DNOP	Saillenfait <i>et al.</i> , 2011	SD rat	GD 6 - 20, gavage	1000	250	Parents: increased liver weight of female
	Poon <i>et al.</i> , 1997	SD rat	13 weeks, gavage	350-402	36-40	Adults: histopathological changes in the liver of male and female

Upper line of each PE shows the DART test data and lower line shows the general toxicity test data.

a) duration, route

b) NOAEL could not be set because of the lowest dose.

GD, gestation day; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level; PND, postnatal day

Comparison of toxicity effects of phthalate esters

ing they provided reliable information. For the hepatotoxicity, we compared the DART and the general toxicity test data separately because the test designs were different for the two types of tests. In this study, we adopted LOAEL and NOAEL of the general toxicity in the generation reproduction toxicity test used for comparing hepatotoxicity. In Table 7, the upper line of each PE shows the DART test data, and lower line shows the general toxicity test data. For the DART test data, DEHP showed the lowest NOAEL of 14 mg/kg/day in the 3-generations toxicity test, based on the increase of liver weights and histopathological changes of male and female. The NOAEL of DEHP was between one-quarter and one-twentieth that of the NOAEL of other PEs (BBP, DBP, DIDP, and DNOP). On the other hand, for the general toxicity test data, DINP showed the lowest NOAEL of 15-18 mg/kg/day in the 2-year carcinogenicity test, based on the increase of male and female liver weight. The NOAEL of DINP was between one-quarter and one-tenth that of the NOAEL of other PEs (BBP, DBP, DIDP, and DNOP). In both test types, liver weight gain was frequently observed in 6 PEs. Among the 6 PEs, only the NOAEL of DBP provided an indication of the influence on the liver of offspring. DBP showed the NOAEL of 133 mg/kg/day, based on the liver weight gain of female offspring. It is known that infants are exposed to PEs through breast milk (Dostal *et al.*, 1987). In the developmental test for DBP, the same dose administered maternally was provided for 4 weeks after weaning, so that the effects of both direct and indirect exposure were considered. The dose was also equivalent to the NOAEL of the 3-month repeated dose toxicity test, so that any difference could not be attributed as dependent on the test design. In the developmental toxicity test, DNOP showed the NOAEL of 250 mg/kg/day, based on the female liver weight gain. On the other hand, in the 13-week repeated dose toxicity test, DNOP showed the NOAEL of 36-40 mg/kg/day, based on the histopathological changes in the liver of male and female. Although there was no difference in the LOAEL or the NOAEL between the DART test and the general toxicity test in other PEs (BBP, DBP, DEHP, DIDP, and DINP), the NOAEL of DNOP in the repeated dose toxicity test was lower than that of the developmental toxicity test. We could not address the reason of this unique behavior of DNOP from the limited information.

To summarize hepatotoxicity, the NOAELs of DEHP and DINP were the lowest among 6 PEs. Regardless of test type, the difference in side chain length does not appear to affect hepatotoxicity. In particular, the hepatotoxicity of BBP and DBP was weaker than that of PEs with long side chain (DIDP, DINP, DNOP). In addition,

test results suggest that DIDP, DINP, and DNOP elicit more hepatotoxicity than DART. For hepatotoxicity, the range of values of the LOAEL and the NOAEL of the 6 PEs was narrow, and was not related to the length of the side chain. Thus, it seems that hepatotoxicity and the effects on the male offspring reproductive organs are different. In rodents, the mechanism of hepatotoxicity of several PEs may involve peroxisome proliferation through PPAR α . Recently, toxic pathways other than PPAR α have been reported; hepatotoxicity is likely to involve a complex mechanism (IARC, 2013).

In conclusions, in this study, we used the published open toxicity test data of 6 PEs to construct a database of toxicity characteristics from the DART information and then organized the hepatotoxicity information. Our database contains structural information, toxicity results, reliability index of results, test information, citation sources of toxicity tests, and a key for identifying chemical substances; moreover, these data are organized in an easy-to-process format, such as Excel and FileMaker. This database can be used to comprehensively and efficiently compare the toxicity effects that are due to differences in the side chain of PEs. By comparing the toxicities 6 kinds of PEs with different side chains on rats, we concluded that DART (especially on male reproductive organs and reproductive ability) and hepatotoxicity showed different toxicities with respect to the length of the side chain. Furthermore, in considering DART, the relevance of the side chain length was different for each detailed endpoint. PEs with C4 - C6 side chains were highly effective on male reproductive organs of offspring and the reproductive ability. However, effects on female reproductive organs and teratogenicity did not depend on side chain length. Moreover, hepatotoxicity was not related to side chain length. Regarding the DART and hepatotoxicity with complicated and wide toxicities, comprehensive and efficient analysis of PEs using the database will be a more useful method in the category approach by increasing the available information in the future. It also makes possible a more detailed grouping of PEs and may be useful for toxicity evaluation of other PEs.

ACKNOWLEDGMENTS

This study was supported by a grant from the Food Safety Commission, Cabinet Office, Government of Japan (Research program for Risk Assessment Study on Food Safety 1602 and 1801). The authors thank Dr. Makoto Ema, Dr. Takashi Yamada, and Mr. Kazuo Ushida for advice on database construction.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Aso, S., Ehara, H., Miyata, K., Hosyuyama, S., Shiraishi, K., Umano, T. and Minobe, Y. (2005): A two-generation reproductive toxicity study of butyl benzyl phthalate in rats. *J. Toxicol. Sci.*, **30**, 39-58.
- Christiansen, S., Boberg, J., Axelstad, M., Dalgaard, M., Vinggaard, A.M., Metzдорff, S.B. and Hass, U. (2010): Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *Reprod. Toxicol.*, **30**, 313-321.
- Coldham, N.G., Dave, M., Sivapathasundaram, S., McDonnell, D.P., Connor, C. and Sauer, M.J. (1997): Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect.*, **105**, 734-742.
- Dostal, L.A., Weaver, R.P. and Schwetz, B.A. (1987): Transfer of di(2-ethylhexyl) phthalate through rat milk and effects on milk composition and the mammary gland. *Toxicol. Appl. Pharmacol.*, **91**, 315-325.
- Ema, M., Itami, T. and Kawasaki, H. (1992): Teratogenic evaluation of butyl benzyl phthalate in rats by gastric intubation. *Toxicol. Lett.*, **61**, 1-7.
- Ema, M., Amano, H., Itami, T. and Kawasaki, H. (1993): Teratogenic evaluation of di-n-butyl phthalate in rats. *Toxicol. Lett.*, **69**, 197-203.
- EU RAR (2003): 1,2-benzenedicarboxylic acid, di-C9-11-branched alkyl esters, C10-rich and di-"isodecyl" phthalate (DIDP). Summary Risk Assessment Report.
- Fabjan, E., Hulzebos, E., Mennes, W. and Piersma, A.H. (2006): A category approach for reproductive effects of phthalates. *Crit. Rev. Toxicol.*, **36**, 695-726.
- Gray, L.E. Jr., Ostby, J., Furr, J., Price, M., Veeramachaneni, D.N. and Parks, L. (2000): Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol. Sci.*, **58**, 350-365.
- Gray, L.E. Jr., Ostby, J., Furr, J., Wolf, C.J., Lambright, C., Parks, L., Veeramachaneni, D.N., Wilson, V., Price, M., Hotchkiss, A., Orlando, E. and Guillelte, L. (2001): Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum. Reprod. Update*, **7**, 248-264.
- Guyton, K.Z., Chiu, W.A., Bateson, T.F., Jinot, J., Scott, C.S., Brown, R.C. and Caldwell, J.C. (2009): A reexamination of the PPAR-alpha activation mode of action as a basis for assessing human cancer risks of environmental contaminants. *Environ. Health Perspect.*, **117**, 1664-1672.
- Hellwig, J., Freudenberger, H. and Jäckh, R. (1997): Differential prenatal toxicity of branched phthalate esters in rats. *Food Chem. Toxicol.*, **35**, 501-512.
- Hushka, L.J., Waterman, S.J., Keller, L.H., Trimmer, G.W., Freeman, J.J., Ambroso, J.L., Nicolich, M. and McKee, R.H. (2001): Two-generation reproduction studies in Rats fed di-isodecyl phthalate. *Reprod. Toxicol.*, **15**, 153-169.
- IARC. (2000): Di(2-ethylhexyl) phthalate. IARC Monogr. Eval. Carcinog. Risks Hum., **77**, 41-148.
- IARC. (2013): Di(2-ethylhexyl)phthalate. IARC Monogr. Eval. Carcinog. Risks Hum., **101**, 149-284.
- ICH. (2005): Detection of toxicity to reproduction for medicinal products & toxicity to male fertility S5 (R2). ICH Harmonised Tripartite Guideline.
- Ito, Y., Yamanoshita, O., Asaeda, N., Tagawa, Y., Lee, C.H., Aoyama, T., Ichihara, G., Furuhashi, K., Kamijima, M., Gonzalez, F.J. and Nakajima, T. (2007): Di(2-ethylhexyl)phthalate induces hepatic tumorigenesis through a peroxisome proliferator-activated receptor alpha-independent pathway. *J. Occup. Health*, **49**, 172-182.
- Klimisch, H.J., Andreae, M. and Tillmann, U. (1997): A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. *Regul. Toxicol. Pharmacol.*, **25**, 1-5.
- Koizumi, M., Ema, M., Hirose, A. and Hasegawa, R. (2000): Recent studies on toxic effects of phthalate esters on reproduction and development: focus on di(2-ethylhexyl)phthalate and di-n-butyl phthalate. *Jpn. J. Food Chem.*, **7**, 65-73. (in Japanese)
- Koizumi, M., Ema, M., Hirose, A., Kurokawa, Y. and Hasegawa, R. (2001): No observed adverse effect levels of phthalate esters on reproductive and developmental toxicity, the differences with age and species in testicular toxicity, and tolerable daily intake of DEHP. *Jpn. J. Food Chem.*, **8**, 1-10. (in Japanese)
- Lake, B.G., Phillips, J.C., Linnell, J.C. and Gangolli, S.D. (1977): The *in vitro* hydrolysis of some phthalate diesters by hepatic and intestinal preparations from various species. *Toxicol. Appl. Pharmacol.*, **39**, 239-248.
- Lee, K.Y., Shibutani, M., Takagi, H., Kato, N., Takigami, S., Uneyama, C. and Hirose, M. (2004): Diverse developmental toxicity of di-n-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology*, **203**, 221-238.
- Lington, A.W., Bird, M.G., Plutnick, R.T., Stubblefield, W.A. and Scala, R.A. (1997): Chronic toxicity and carcinogenic evaluation of diisononyl phthalate in rats. *Fundam. Appl. Toxicol.*, **36**, 79-89.
- Marsman, D. (1995): NTP (National Toxicology Program) technical report on toxicity studies of dibutyl phthalate (CAS No. 84-74-2) administered in feed to F344/N Rats and B6C3F1 mice. *Toxic. Rep. Ser.*, **30**, 1-107.
- Masutomi, N., Shibutani, M., Takagi, H., Uneyama, C., Takahashi, N. and Hirose, M. (2003): Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. *Toxicology*, **192**, 149-170.
- McIntyre, B.S., Barlow, N.J. and Foster, P.M. (2001): Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol. Sci.*, **62**, 236-249.
- Mitchell, F.E., Price, S.C., Hinton, R.H., Grasso, P. and Bridges, J.W. (1985): Time and dose-response study of the effects on rats of the plasticizer di(2-ethylhexyl) phthalate. *Toxicol. Appl. Pharmacol.*, **81**, 371-392.
- Mylchreest, E., Cattley, R.C. and Foster, P.M. (1998): Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol. Sci.*, **43**, 47-60.
- Mylchreest, E., Sar, M., Cattley, R.C. and Foster, P.M. (1999): Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.*, **156**, 81-95.
- NAFTA. (2012): (Quantitative) Structure Activity Relationship [(Q) SAR] guidance document.
- Nagao, T., Ohta, R., Marumo, H., Shindo, T., Yoshimura, S. and Ono, H. (2000): Effect of butyl benzyl phthalate in Sprague-Dawley rats after gavage administration: a two-generation reproductive study. *Reprod. Toxicol.*, **14**, 513-532.

Comparison of toxicity effects of phthalate esters

- NTP. (1997): Toxicology and carcinogenesis studies of butyl benzyl phthalate (CAS No. 85-68-7) in F344/N rats (feed studies). Natl Toxicol Program Tech Rep Ser., **No. 458**.
- NTP. (2003): NTP-CERHR monograph on the potential human reproductive and developmental effects of di-n-butyl phthalate (DBP). NTP. CHRHR. MON.
- OECD. (2008): Guidance document on mammalian reproductive toxicity testing and assessment. OECD Series on Testing and Assessment, **No. 43**.
- OECD. (2014): Guidance on grouping of chemicals, second edition. OECD Series on Testing and Assessment, **No. 194**.
- OECD. (2018): Test No. 414: Prenatal developmental toxicity study. OECD Guidelines for the Testing of Chemicals, Section 4: Health effects.
- Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J. and Gray, L.E. Jr. (2000): The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci.*, **58**, 339-349.
- Poon, R., Lecavalier, P., Mueller, R., Valli, V.E., Procter, B.G. and Chu, I. (1997): Subchronic oral toxicity of di-n-octyl phthalate and di(2-Ethylhexyl) phthalate in the rat. *Food Chem. Toxicol.*, **35**, 225-239.
- Saillenfait, A.M., Roudot, A.C., Gallissot, F. and Sabaté, J.P. (2011): Prenatal developmental toxicity studies on di-n-heptyl and di-n-octyl phthalates in Sprague-Dawley rats. *Reprod. Toxicol.*, **32**, 268-276.
- Tyl, R.W., Myers, C.B., Marr, M.C., Fail, P.A., Seely, J.C., Brine, D.R., Barter, R.A. and Butala, J.H. (2004): Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. *Reprod. Toxicol.*, **18**, 241-264.
- Ward, J.M., Peters, J.M., Perella, C.M. and Gonzalez, F.J. (1998): Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator-activated receptor α -null mice. *Toxicol. Pathol.*, **26**, 240-246.
- Waterman, S.J., Ambroso, J.L., Keller, L.H., Trimmer, G.W., Nikiforov, A.I., Harris, S.B. (1999): Developmental toxicity of di-isodecyl and di-isononyl phthalates in rats. *Reprod. Toxicol.*, **13**, 131-136.
- Waterman, S.J., Keller, L.H., Trimmer, G.W., Freeman, J.J., Nikiforov, A.I., Harris, S.B., Nicolich, M.J. and McKee, R.H. (2000): Two-generation reproduction study in rats given di-isononyl phthalate in the diet. *Reprod. Toxicol.*, **14**, 21-36.
- Wine, R.N., Li, L.H., Barnes, L.H., Gulati, D.K. and Chapin, R.E. (1997): Reproductive toxicity of di-n-butylphthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ. Health Perspect.*, **105**, 102-107.
- Wolf, C. Jr., Lambright, C., Mann, P., Price, M., Cooper, R.L., Ostby, J. and Gray, L.E. Jr. (1999): Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health*, **15**, 94-118.
- Wolfe, G.W. and Layton, K.A. (2004): Diethylhexylphthalate: multigenerational reproductive assessment by continuous breeding when administered to Sprague-Dawley rats in the diet. TherImmune Research Corporation (TRC) study No. 7244-200, NTP-RACB 98-004.
- Zacharewski, T.R., Meek, M.D., Clemons, J.H., Wu, Z.F., Fielden, M.R. and Matthews, J.B. (1998): Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. *Toxicol. Sci.*, **46**, 282-293.