Figure 2: Comparison of change in relative liver weight of male and female intact and castrated rats given HDBB by gavage.
[a]The result of the previous 28-day repeated dose toxicity study, in which male and female intact rats were given HDBB once-daily at 0 (vehicle control), 0.5, 2.5, 12.5, and 62.5 mg/kg/day by gavage (Hirata-Koizumi et al., 2007)

The current study showed that the gender-related difference in susceptibility to HDBB hepatotoxicity was reduced, but not abolished, by castration. Sexual differences found in the present study were considered to be due to exposure to sexual hormones before four weeks of age, when castration was conducted. In female rats, serum estradiol concentration during the first three weeks after birth is as high as or higher than the level during the proestrus stage in young adults (Döhler and Wuttke, 1975); however, because serum estradiol concentration is similarly high during this preweaning period in male rats, it is unlikely that exposure to estradiol during this period contributes to the sexual difference in susceptibility of rats to the toxicity of HDBB. On the other hand, serum androgen levels before four weeks of age are much higher in male than female rats (Döhler and Wuttke, 1975). Ketelslegers et al. (1978) reported that plasma testosterone level in male rats was as high as 50 ng/100 mL two days after birth and it remained at the same level until day 8. The progressive decline occurred from days 8–24, and the testosterone level remained low, at the limit of detection of the assay (18 ng/ 100 mL), until day 30. There is a possibility that neonatal exposure to testosterone plays some role in the different susceptibility of male and female rats to the toxicity of HDBB. In fact, neonatal exposure to androgen irreversibly programs brain centers involved in the hypothalamo-pituitary control of hepatic sex-dependent metabolism (Gustafsson et al., 1981). We are currently in the process of performing a repeated dose toxicity study of HDBB using preweaning rats to clarify when gender-related differences in susceptibility to the toxicity of HDBB develop.

As in the case of HDBB, the male-predominant induction of toxicity in rats has been reported for many other substances, such as adenine (Ogirima et al.,

2006), acetaminophen (Raheja et al., 1983), dapsone (Coleman et al., 1990), fluoranthene (Knuckles et al., 2004), 3-nitropropionic acid (Nishino et al., 1998), and mercuric chloride (Muraoka and Itoh, 1980). Various causes of such gender-related differences are indicated mainly for toxicokinetic determinants. It is well known that hepatic metabolism differs between the sexes, with male rats generally having higher activity than females (Gad, 2006). Furthermore, gender differences in membrane transport in various organs, including the kidneys, liver, intestine, and brain, have emerged relatively recently (Morris et al., 2003). In the case of HDBB, male rats consistently showed greater susceptibility to various effects of HDBB (e.g., on the liver, blood, etc.) in the previous 28-day and 52-week studies (Hirata-Koizumi et al., 2007; 2008); therefore, such differences in metabolism or transport between the sexes might increase the blood concentration of causative substances (i.e., HDBB or its metabolites) in males.

For gender-related variations in toxicokinetic determinants, many mechanistic studies on the metabolic enzyme cytochrome P450 have been reported (Waxman and Chang, 2005). In rats, a subset of P450s is expressed in a sex-dependent fashion and is subject to endocrine control. Whereas testosterone has a major positive regulatory influence on male-specific P450 forms, estrogen plays a somewhat lesser role in the expression of the female-specific/predominant liver P450 enzymes. If the male-specific/predominant metabolic enzymes have an intimate involvement in the toxic activation of HDBB, our results, showing the higher susceptibility of male rats to HDBB toxicity than females and decreased susceptibility by castration of male rats, could be explained. Interestingly, it was reported that estradiol suppressed the expression of male-specific/predominant P450 enzymes (Waxman and Chang, 2005). This is consistent with our results that female susceptibility to the hepatotoxicity of HDBB was increased by castration, given that the male-specific/predominant P450 enzymes activate HDBB. Since the expression of female-specific/predominant P450 enzymes is reduced by testosterone treatment as well as by castration of females (Waxman and Chang, 2005), there is also the possibility that these enzymes might be involved in the detoxication of HDBB. In order to clarify the cause of the sexual differences in susceptibility of rats to the toxicity of HDBB, we are planning a toxicokinetic study of HDBB, which would include the identification of metabolites and the related metabolic enzyme as well as measurement of the blood concentration of HDBB both after a single and repeated administration of HDBB to rats.

CONCLUSIONS

The current results showed that an oral administration of HDBB to castrated rats for 28 days caused hepatotoxicity at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. Castration markedly reduced gender-related differences in the toxicity of HDBB in male and female rats.

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ELSEVIER

Reproductive and developmental toxicity screening test of tetrahydrofurfuryl alcohol in rats

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Abstract

Twelve male and female rats per group were given tetrahydrofurfuryl alcohol (THFA) by gavage at 0, 15, 50, 150 or 500 mg/kg/day. Males were dosed for 47 days, beginning 14 days before mating, and females were dosed for 42–52 days beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. Changes in locomotor activity, inhibition of body weight gain, and/or histopathological changes in the thymus, spleen, testes and/or epididymides were observed in males and females at 150 mg/kg and above. No effects of THFA were found on the copulation index, fertility index, or the number of corpora lutea and implantations in pregnant females. At 500 mg/kg, no pregnant females delivered any pups. At 150 mg/kg, gestation length was prolonged, and the total number of pups born and the number of live pups on postnatal days 0 and 4 was markedly decreased. No effects of THFA were found on the sex ratio and body weight of live pups, or the incidence of pups with malformations or variations. Based on these findings, the NOAELs for parental and reproductive/developmental toxicity of THFA were concluded to be 50 mg/kg/day in rats.
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Keywords: Tetrahydrofurfuryl alcohol; Reproductive and developmental toxicity; Postimplantation loss; Postnatal loss; Testicular toxicity; Rat

1. Introduction

Tetrahydrofurfuryl alcohol (THFA; CAS No. 97-99-4) is a colorless and flammable liquid with a slight ether odor [1]. In Japan, the annual production and import volume of THFA was reported to be from 100 to 1000 tonnes in 2004 [2], but there is no data available on that in other countries. The major uses of this chemical are as a solvent for various products (fats, waxes, resins, dyes and others) and as an intermediate in industrial applications [1]. While the extensive use of THFA by industry creates significant potential for occupational exposure, there is also the possibility of exposure of the general population to THFA because some of the applications include consumer uses, such as floor polish removers, graffiti removers and oven cleaners [3]. In particular, THFA application as a solvent for nail-cleaning

agents [1] and absorption enhancer in various lotions and transdermal medications [4] would cause relatively high levels of exposure due to direct use on the skin. Such occupational and consumer exposure could occur through inhalation and dermal routes. On the other hand, THFA is directly added to food as a flavoring agent in Japan [5], and its use as a food additive for flavoring is also permitted in the US [6] and EU [7]. Furthermore, this chemical is known as the "solvent of choice" for a variety of agricultural applications, including pest control, weed control and growth regulation [3]. These uses suggest possible exposure of the general population to THFA via food. For each application, there are no data available on the actual use volume and exposure levels at this time. The possibility of human exposure to THFA has aroused concern regarding its toxicological potential.

Only limited information is available about the toxicity of THFA. It was reported that oral LD_{50} was 1.6–3.2 g/kg in rats and 0.8–1.6 g/kg in guinea pigs, and inhalation exposure for 6 h caused 2/3 deaths of rats at 12,650 ppm [8]. THFA showed eye irritation in rabbits [9] but did not irritate mouse skin [10].

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Unpublished repeated dose toxicity data are briefly summarized in OECD SIDS (Screening Information Data Set) documents [1]. In a 90-day feeding study using rats, body weight gain was depressed at 1000 ppm and above, the relative weight of epididymides decreased at 5000 ppm and above, and relative testis weight decreased with moderate testicular degeneration accompanied with complete loss of spermatogenic activity observed at 10,000 ppm. Adverse effects on body weight gain and male reproductive organs were also found in a 90-day inhalation and dermal study of THFA using rats. As for reproductive and developmental toxicity, only a dose range-finding developmental toxicity study is available [11]. In rats given THFA by gavage on days 6–15 of pregnancy, total embryonic loss occurred in all females at 500 mg/kg and above, at which inhibition of maternal body weight gain was also observed. Fetuses with a filamentous tail (5/124 fetuses) and lowering of fetal weight were found at 100 mg/kg without maternal toxicity.

Since there is insufficient information on toxicity, this chemical was selected as an object substance in an existing chemical testing program by the Japanese government [12]. In this program, a reproduction/developmental toxicity screening test was performed according to OECD test guideline 421 [13], because the evaluation of reproductive and developmental toxicity is essential in the risk assessment of chemicals. The results are summarized in OECD SIDS documents [1] and an assessment report prepared by US EPA, "Hazard assessment for the tolerance reassessment of tetrahydrofurfuryl alcohol (THFA)" [14]; however, detailed data have not been published in scientific journals. In this paper, therefore, we reported the data of a reproduction/developmental toxicity screening test of THFA.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [13], and in accordance with the principles for Good Laboratory Practice [15,16] at the Research Institute for Animal Science in Biochemistry & Toxicology (Sagamihara, Japan). The experiment was approved by the Animal Care and Use Committee of the Research Institute for Animal Science in Biochemistry & Toxicology, and was performed in accordance with the ethics criteria contained in the bylaws of the Committee.

2.1. Animals and housing conditions

Crj:CD(SD)IGS rats (SPF, 8 weeks old) were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimatized to the laboratory for 13 days and subjected to treatment at 10 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. In addition, vaginal smears of each female were recorded, and only females showing a 4- to 5-day estrous cycle were used in the experiment. On the day before initial treatment, the rats were distributed into 5 groups of 12 males and 12 females each by stratified random sampling based on body weight.

Throughout the study, animals were maintained in an air-conditioned room at 21.9–22.4 °C, with a relative humidity of 49–57%, a 12-h light/dark cycle, and ventilation with more than 10 air changes/h. A basal diet (Labo MR Stock; Nosan Corporation, Yokohama, Japan) and sterile water were provided *ad libitum*. They were housed individually, except for mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and/or litters were reared using wood chips as bedding (White Flake; Charles River Japan, Inc., Yokohama, Japan).

2.2. Chemicals and doses

THFA was obtained from Koatsu Chemical Industries, Ltd. (Osaka, Japan) and kept in a cool (4 °C) and dark place. The THFA (Lot no. 2002–4) used in this study was 99.5% pure, and stability during the study was verified by gas chromatography. The test article was dissolved in purified water (Kyouei Pharmaceutical Co. Ltd., Takaoka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once a week and kept in a cool (4 °C) and dark place until dosing, as stability under these conditions has been confirmed for up to 7 days. The concentrations of THFA in the formulations were confirmed to be 97.7–103.0% of the target by gas chromatography analysis.

Prior to the present reproductive and developmental toxicity screening study, a 14-day dose-finding study was performed. In the dose-finding study, male and female rats were given THFA by gavage at 50, 100, 200, 500 or 1000 mg/kg/day for 14 days. Changes in locomotor activity were observed at 100 mg/kg and above, decreases in absolute and relative weight of the pituitary and thymus were detected at 200 mg/kg and above, and piloerection, decrease in food consumption and dilatation of the cecum were found at 500 mg/kg and above (data not shown). Taking into account the results of this dose-finding study, the dose levels of THFA in the present study were set as 15, 50, 150 or 500 mg/kg/day. The daily application volume (5 ml/kg body weight) was calculated according to the latest body weight.

2.3. Study design

Male rats were dosed once daily for 47 days, beginning 14 days before mating and throughout the mating period. Female rats were also dosed once daily from 14 days prior to mating, and throughout the mating and gestation periods, to day 4 of lactation. The total administration period was 42–52 days. The day of the first dosing was designated as day 0 of the administration/premating period.

During the first 14-day administration period (premating period), vaginal lavage samples of each female were evaluated daily for estrous cyclicity. After this premating period, female rats were transferred to the home cage of a male of the same group, and cohabited on a 1:1 basis until successful copulation occurred or the mating period of 2 weeks had elapsed. During the mating period, vaginal smears were examined daily for the presence of sperm, and the presence of sperm in the vaginal smear and/or a vaginal plug were considered as evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. Pregnant females were allowed to deliver spontaneously and nurse their pups, and the day on which parturition was completed by 9:30 was designated as day 0 of lactation or postnatal day (PND) 0.

Throughout the study, all parental animals were observed for clinical signs of toxicity at least twice a day. The body weight was recorded on days 0, 7, 14, 21, 28, 35, 42 and 46 of the dosing period in males, and on days 0, 7 and 14 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 4 of the lactation period in females. Food consumption was recorded on days 0, 7, 21, 28, 35, 42 and 45 of the dosing period in males, and on days 0 and 7 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 3 of the lactation period in females.

All surviving male rats were euthanized by exsanguination under ether anesthesia on the day after the last administration. All female rats showing successful reproductive performance were euthanized in a similar way on day 5 of lactation. Females that did not copulate were euthanized on the day after the 52nd administration. Females that had not completed parturition were euthanized 5 days after the expected day of parturition (day 22 of gestation). When total litter loss was observed, the dams were euthanized within 4 days. For all parental animals, the external surfaces were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. For females, the numbers of corpora lutea and implantation sites were recorded. In males, the testes and epididymides were removed and weighed. The pituitary, thymus and kidneys were also weighed in both sexes.

Histopathological evaluations were performed on the pituitary, thymus, testes, epididymides and ovaries of all animals in the control and highest dose groups. In addition, the spleen of five animals in the control group and of all animals in the highest dose group was examined as test substance-related changes were macroscopically found in this organ. As a result of histopathological examination, test substance-related changes were found in the thymus,

spleen, testes and epididymides of the highest dose group; therefore, the organs of five animals in the other groups were also examined histopathologically. For females that showed reproductive failure, the pituitary, ovaries, uterus and/or mammary gland were examined histopathologically. For the histopathological examination, the target organs were fixed in 10% neutral-buffered formalin (following Bouin's fixation for the testes and epididymides), processed routinely for embedding in paraffin, and sections were prepared for staining with hematoxylin–eosin.

All live and dead pups were counted, and live pups were sexed, examined grossly and weighed on PND 0. They were daily observed for clinical signs of toxicity on PNDs 0–4. On PND 4, the number and body weight of live pups was recorded. The pups were then euthanized by exsanguination under ether anesthesia, and gross internal examinations were performed.

2.4. Data analysis

Parametric data, such as body weight, food consumption, organ weight, gestation length and the number of corpora lutea, implantations and pups born, were analyzed by Bartlett's test for homogeneity of distribution. When homogeneity was recognized, one-way analysis of variance was performed. If a significant difference was detected, Sheffé's test was conducted for comparisons between control and individual treatment groups. Data without homogeneity or some non-parametric data (implantation index, live birth index, delivery index, variability index, the incidence of pups with malformations or variations) were analyzed using the Kruskal–Wallis's rank sum test. If significant differences were found, the mean rank test of Scheffé's type was conducted for comparison between the control and each dosage group.

For toxicological signs, autopsy results and histopathological findings, Fisher's exact test was conducted for comparison of the incidences in each group. The sex ratio of live pups was also compared by Fisher's exact test. The copulation index, fertility index and gestation index were compared using the χ^2-test.

Pups were statistically analyzed using the litter as the experimental unit. The 5% level of probability was used as the criterion for significance.

3. Results

3.1. Parental toxicity

One male of the 15 mg/kg group was found dead after the 22nd administration. No substance-related clinical signs of toxicity were detected at 15 and 50 mg/kg. Increase and decrease in locomotor activity was observed in 10/12 males and 11/12 females in the 150 mg/kg group and in all animals of the 500 mg/kg group. This change was found mainly in the first half of the administration period in both sexes at 150 mg/kg and in females at 500 mg/kg, and also in the second half of the administration period in males at 500 mg/kg. Vaginal hemorrhage was observed during the late gestation period in 1/11 pregnant female at 150 mg/kg and 2/12 pregnant females at 500 mg/kg, which did not deliver their pups or experienced total litter loss.

Table 1
Body weight of male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
Males (no. = 12)					
Body weight during administration (g)					
Day 0	393 ± 17	394 ± 17	393 ± 14	392 ± 17	392 ± 16
Day 7	422 ± 23	420 ± 18	421 ± 16	419 ± 22	400 ± 18*
Day 14	448 ± 28	441 ± 21	445 ± 18	444 ± 24	424 ± 21.
Day 21	470 ± 28	459 ± 29	469 ± 19	466 ± 24	443 ± 19*
Day 28	492 ± 31	482 ± 22	488 ± 21	482 ± 21	458 ± 22*
Day 35	516 ± 34	506 ± 24	510 ± 25	491 ± 22	472 ± 28**
Day 42	536 ± 38	524 ± 29	523 ± 28	505 ± 21	482 ± 31**
Day 46	550 ± 40	532 ± 29	533 ± ±27	513 ± 21	489 ± 32**
Gain	157 ± 29	136 ± 19	140 ± 25	122 ± 16*	98 ± 23**
Females (no. = 12)					
Body weight during premating (g)					
Day 0	236 ± 15	234 ± 13	232 ± 14	235 ± 16	234 ± 14
Day 7	249 ± 14	244 ± 13	241 ± 14	243 ± 20	242 ± 15
Day 14	265 ± 18	255 ± 15	252 ± 18	260 ± 21	256 ± 16
Gain	29 ± 10	21 ± 7	20 ± 10	25 ± 9	22 ± 10
Body weight during gestation (g)					
Day 0	275 ± 23	266 ± 19	261 ± 18	259 ± 20	262 ± 20
Day 7	317 ± 24	304 ± 25	300 ± 23	301 ± 21	297 ± 18
Day 14	357 ± 23	339 ± 26	335 ± 27	332 ± 21	322 ± 20*
Day 20	438 ± 23	422 ± 31	411 ± 34	373 ± 27**	320 ± 20**
Gain	164 ± 9	156 ± 15	150 ± 18	114 ± 20*	58 ± 8**
Body weight during lactation (g)					
Day 0	343 ± 19	327 ± 28	321 ± 26	308 ± 17	
Day 4	361 ± 22	351 ± 34	341 ± 28	306	
Gain	18 ± 12	24 ± 13	20 ± 9	3	

Values are given as the mean ± S.D.
* Significantly different from the control group ($P < 0.05$).
** Significantly different from the control group ($P < 0.01$).

Body weight and the gain in each group are shown in Table 1. In the 500 mg/kg group, body weight was significantly reduced on day 7 and from day 21 to the end of the dosing period in males. In females, significant reduction of body weight was found on day 20 of gestation at 150 mg/kg and on days 14 and 20 of gestation at 500 mg/kg. Body weight gain during the whole period of administration in males and during the gestation period in females was significantly decreased in the 150 and 500 mg/kg groups.

Food consumption was significantly decreased on day 21 of the administration period at 50 mg/kg, on day 7 of the administration period at 150 mg/kg and on days 0, 7 and 21 of the administration period at 500 mg/kg in males, and on days 14 and 20 of the gestation period at 150 mg/kg and on day 0 of the premating period and days 0, 14 and 20 of the gestation period at 500 mg/kg in females (data not shown).

At necropsy, the incidence of small-sized thymus, testes and epididymides was significantly increased at 500 mg/kg in males. Significant increase in the incidence of a rough surface and white spots in the spleen was also found in both sexes of the 500 mg/kg group (data not shown).

Absolute and relative organ weight of scheduled-sacrifice animals in each group is shown in Table 2. Absolute pituitary weight was significantly decreased at 150 mg/kg and above in both sexes. Absolute and relative weight of the thymus, testes and epididymides were also significantly decreased in males of the 500 mg/kg group. In addition, significant decreases in absolute kidney weight at 500 mg/kg in males, and increases in the relative kidney weight at 150 mg/kg in females were detected.

On histopathology, test substance-related changes were observed in the thymus, spleen, testes and epididymides, as shown in Table 3. In the thymus, the incidence of atrophy was significantly increased at 500 mg/kg in males. In the spleen, the incidence of capsule inflammation was significantly increased at 500 mg/kg in both sexes, and the grade of extramedullary hematopoiesis was significantly decreased at 150 mg/kg and above in females. Significant increases in the incidence of seminiferous tubular atrophy and hyperplasia of interstitial cells in the testes, and cell debris and decreased sperm in the lumen of epididymides were also detected in males of the 500 mg/kg group.

3.2. Reproductive findings

The reproductive findings in rats given THFA are presented in Table 4. An estrous cycle of over 5 days was observed in only one female each in the control, 150 and 500 mg/kg groups, but the mean estrous cycle at 500 mg/kg was significantly prolonged. One pair at 15 mg/kg did not copulate and the male was found dead on day 7 of the mating period. One female each at 15 and 150 mg/kg did not become impregnated. The copulation index, precoital interval and fertility index were not significantly different between the control and THFA-treated groups. All pregnant females at 500 mg/kg and two of 11 pregnant females at 150 mg/kg did not deliver any pups. In these females, total early resorption (1/2 females at 150 mg/kg and 12/12 females at 500 mg/kg) or mummification of all fetuses (1/2 females at 150 mg/kg) were found in the uterus. In the 150 mg/kg group, the

Table 2

Organ weight of male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of males	12	11	12	12	12
Body weight (g)	550 ± 40	535 ± 30	538 ± 28	517 ± 22	489 ± 33[**]
Pituitary (mg)	15.6 ± 1.5	15.6 ± 2.0	14.2 ± 1.3	13.4 ± 1.5[*]	12.2 ± 1.2[**]
	(2.8 ± 0.3)	(2.9 ± 0.4)	(2.7 ± 0.3)	(2.6 ± 0.3)	(2.5 ± 0.2)
Kidneys (g)	3.10 ± 0.18	3.15 ± 0.32	3.09 ± 0.20	2.90 ± 0.20	2.71 ± 0.20[**]
	(0.57 ± 0.04)	(0.59 ± 0.07)	(0.58 ± 0.05)	(0.56 ± 0.03)	(0.55 ± 0.03)
Thymus (g)	0.36 ± 0.07	0.32 ± 0.06	0.35 ± 0.06	0.31 ± 0.07	0.19 ± 0.05[**]
	(0.07 ± 0.01)	(0.06 ± 0.01)	(0.07 ± 0.01)	(0.06 ± 0.01)	(0.04 ± 0.01[**])
Testes (g)	3.41 ± 0.50	3.18 ± 0.83	3.52 ± 0.29	3.40 ± 0.45	1.77 ± 0.44[**]
	(0.63 ± 0.11)	(0.60 ± 0.15)	(0.66 ± 0.07)	(0.66 ± 0.10)	(0.36 ± 0.09[**])
Epididymides (g)	1.40 ± 0.20	1.30 ± 0.30	1.38 ± 0.15	1.26 ± 0.17	0.87 ± 0.15[**]
	(0.26 ± 0.04)	(0.24 ± 0.05)	(0.26 ± 0.03)	(0.24 ± 0.04)	(0.18 ± 0.03[**])
No. of females	12	10	12	9	0
Body weight (g)	363 ± 25	350 ± 35	339 ± 24	313 ± 27[**]	
Pituitary (mg)	20.1 ± 3.8	18.3 ± 1.7	17.6 ± 1.8	16.0 ± 1.9[*]	
	(5.5 ± 0.8)	(5.3 ± 0.3)	(5.2 ± 0.5)	(5.1 ± 0.2)	
Kidneys (g)	2.06 ± 0.19	2.00 ± 0.22	2.06 ± 0.23	1.98 ± 0.25	
	(0.57 ± 0.04)	(0.57 ± 0.06)	(0.61 ± 0.05)	(0.63 ± 0.05[*])	
Thymus (g)	0.30 ± 0.08	0.28 ± 0.09	0.26 ± 0.07	0.22 ± 0.05	
	(0.08 ± 0.02)	(0.08 ± 0.03)	(0.08 ± 0.02)	(0.07 ± 0.01)	

Values are given as the mean ± S.D. Values in parentheses are relative organ weights (g or mg/100 g body weight).

[*] Significantly different from the control group (P < 0.05).

[**] Significantly different from the control group (P < 0.01).

Table 3
Histopathological findings in male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Grade	Dose (mg/kg/day)				
		0	15	50	150	500
Males						
Thymus		(12)	(5)	(5)	(5)	(12)
Atrophy	+	0	0	0	1	8 ⎤ **
	++	0	0	0	0	1 ⎦
Spleen		(5)	(5)	(5)	(5)	(12)
Extramedullary hematopoiesis	+	2	3	3	4	10
	++	3	2	2	0	2
Capsule inflammation	+	0	0	0	3	5 ⎤ **
	++	0	0	0	0	4 ⎥
	+++	0	0	0	0	2 ⎦
Testes		(12)	(5)	(5)	(5)	(12)
Atrophy of seminiferous tubule	+	0	0	0	1	4 ⎤ **
	++	1	0	0	0	7 ⎥
	+++	0	0	0	0	1 ⎦
Hyperplasia of interstitial cells	+	1	0	0	0	9 ⎤ **
	++	0	0	0	0	1 ⎦
Epididymides		(12)	(5)	(5)	(5)	(12)
Decrease in sperm	+	0	0	0	1	3 ⎤ **
	++	1	0	0	0	8 ⎥
	+++	0	0	0	0	1 ⎦
Cell debris in lumen	+	1	0	0	1	3 ⎤ **
	++	0	0	0	0	9 ⎦
Females						
Thymus		(12)	(5)	(5)	(5)	(12)
Atrophy	+	1	0	1	2	4
Spleen		(5)	(5)	(5)	(5)	(12)
Extramedullary hematopoiesis	+	0	0	1	5	11
	++	4	4	4	0 ⎤ **	1 ⎤ **
	+++	1	1	0	0 ⎦	0 ⎦
Capsule inflammation	+	0	0	0	1	5 ⎤ **
	++	0	0	0	1	4 ⎥
	+++	0	0	0	0	3 ⎦

Values represent the number of animals with findings. Values in parentheses are the number of animals examined. +, slight; ++, moderate; +++, severe.
** Significantly different from the control ($P < 0.01$).

remaining nine pregnant females began to deliver on days 24–25 of gestation, but five did not have any pups the next morning. The gestation length in the 150 mg/kg group was significantly prolonged. The gestation index was significantly decreased at 150 mg/kg and above.

3.3. Developmental findings

The developmental findings in rats given THFA are shown in Table 5. No effects of THFA were observed in the number of corpora lutea and implantations, and the implantation index. At 500 mg/kg, no pups were obtained. A significantly decreased total number of pups born, number of live pups on PNDs 0 and 4, and delivery and live birth index, and an increased number of dead pups on PND 0 were found at 150 mg/kg. There was no significant difference in the sex ratio of live pups, the viability index

on PND 4, and body weight of male and female pups on PNDs 0 and 4 between the control and THFA-treated groups. Although one pup with general edema was observed at 150 mg/kg, no significant difference in the incidence of pups with malformation was found. Pups with internal variations, such as thymic remnants in the neck and/or left umbilical artery, were observed in all groups, including the control group; however, the total numbers of pups with internal and individual variations were not significantly increased in any THFA-treated groups.

4. Discussion

The current study was conducted to examine the possible effects of THFA on reproduction and development in rats. The dosage of THFA used in this study was sufficiently high to be expected to induce general toxic effects in parental animals. As

Table 4
Reproductive findings in rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of pairs	12	12	12	12	12
Estrous cycles (day)[a]	4.3 ± 0.6	4.0 ± 0.1	4.1 ± 0.3	4.5 ± 0.6	4.8 ± 0.5[c]
Copulation index (male/female)[b]	100/100	91.7/91.7	100/100	100/100	100/100
No. of pairs with successful copulation	12	11	12	12	12
Precoital interval (day)[a]	2.7 ± 1.2	2.5 ± 1.4	2.9 ± 1.2	2.3 ± 1.4	3.7 ± 2.7
Fertility index [c]	100	90.9	100	91.7	100
No. of pregnant females	12	10	12	11	12
No. of pregnant females with parturition	12	10	12	9	0
Gestation length (day)[a]	22.6 ± 0.5	22.7 ± 0.5	22.9 ± 0.3	24.7 ± 0.7[**]	
Gestation index[d]	100	100	100	36.4[**]	0[**]
No. of dams delivering live pups	12	10	12	4	0

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = no. of copulated rats/no. of pairs × 100.
[c] Fertility index (%) = no. of pregnant females/no. of pairs with successful copulation × 100.
[d] Gestation index (%) = no. of dams with live pups/no. of pregnant females × 100.
[*] Significantly different from the control group ($P < 0.05$).
[**] Significantly different from the control group ($P < 0.01$).

expected, changes in locomotor activity, lowered body weight, and/or histopathological changes in the thymus, spleen, testes and epididymides were observed at 150 mg/kg and above.

Death at 15 mg/kg was considered to be incidental because death occurred in only one male and showed no dose dependency. Also, the decrease in food consumption found in males of the 50 mg/kg group was considered to be toxicologically insignificant because the decrease was transient and was not accompanied with changes in body weight.

In males, body weight gain during the whole administration period was suppressed at 150 and 500 mg/kg, but decreased food consumption was found only during the early administration period at 500 mg/kg and was transient at 150 mg/kg; therefore, factors other than reduced food consumption must be involved in the inhibitive effect of THFA on body weight. In females, the inhibition of body weight gain during the late gestation period at 150 mg/kg and above is considered to be mainly due to the lack of embryos/fetuses because the total number of pups born was markedly decreased in these groups. Similarly, decreased food consumption during the late gestation period is due to decreased nutritional requirement accompanied with embryonic/fetal loss.

Atrophy of the thymus detected at 500 mg/kg in males was accompanied with a marked decrease in organ weight (about 50% of the control value). In addition to these findings, capsule inflammation and/or decreased extramedullary hematopoiesis detected in the spleen of males at 500 mg/kg and of females at 150 mg/kg and above suggests that THFA affects hematological and immunological parameters. Actually, decreased levels of hemoglobin and/or platelet counts were reported in an unpublished 90-day inhalation and feeding study of THFA using rats [1].

Seminiferous tubular atrophy in the testes could be recognized as direct action on the germinal epithelium or secondary change through decreased secretion of gonadotrophic hormone from the pituitary [17]. In the present study, seminiferous tubular atrophy was associated with hyperplasia of interstitial cells,

which develops with increased levels of luteinizing hormone (LH) in rats [17]; therefore, THFA is considered to exert effects directly on the testes and to impair spermatogenesis. THFA might impair testosterone synthesis, leading to increased LH levels via negative feedback. The reduced pituitary weight found in males in the 150 and 500 mg/kg groups might be related to such disruption of the hypothalamus–pituitary–gonadal axis.

Despite such histopathological changes in the testes with decreased sperm number in the epididymides, no effects of THFA on reproductive parameters, such as precoital interval, copulation and fertility index, were observed in the present study. These findings are supported by the following descriptions by Parker [18]. Rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility, particularly as evaluated in reproductive studies that allow multiple mating. It is also reported that sperm production can be drastically reduced (by up to 90% more) without affecting fertility in Sprague–Dawley and Wistar rats [19,20].

The prolonged estrous cycle at 500 mg/kg and decreased pituitary weight at 150 mg/kg in females might also suggest disruption of the hypothalamus–pituitary–gonadal axis; however, because the degree of change in the estrous cycle was slight and most females showed 4- to 5-day estrous cycles, this change is considered to be toxicologically insignificant. Parker [18] noted that estrous cyclicity can be impaired at doses below those that alter fertility, and such changes without associated changes in reproductive or hormonal endpoints would not be considered adverse.

In the current study, total embryonic loss was noted in pregnant females in the higher dose groups. These findings were consistent with the previous developmental toxicity study, in which total embryonic loss was found at 500 mg/kg and above [11]. At 150 mg/kg in the present study, most females showed parturition behavior, but only about half of the dams had pups the next day and the total number of pups born markedly decreased. Cannibalism might have occurred in this group. Even animals

Table 5
Developmental findings in rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of pregnant females	12	10	12	11	12
No. of corpora lutea[a]	17.7 ± 2.1	16.5 ± 2.7	17.8 ± 1.5	16.4 ± 2.0	17.0 ± 2.8
Implantation index[a,b]	88.8 ± 7.4	93.5 ± 7.4	90.7 ± 8.0	84.5 ± 13.1	87.9 ± 23.7
No. of implantation sites[a]	15.6 ± 1.3	15.3 ± 1.9	16.1 ± 1.8	13.7 ± 2.1	14.5 ± 3.7
No. of litters	12	10	12	4	0
Delivery index[a,c]	95.3 ± 7.1	94.7 ± 6.2	91.9 ± 5.9	46.4 ± 14.0[c]	
Total no. of pups born[a]	14.8 ± 1.6	14.5 ± 2.1	14.8 ± 1.7	7.0 ± 1.4[**]	
Live birth index[a,d]	100 ± 0	100 ± 0	98.8 ± 2.8	43.1 ± 29.3[c]	
No. of live pups on PND 0[a]	14.8 ± 1.6	14.5 ± 2.1	14.6 ± 1.8	3.0 ± 2.2[**]	
No. of dead pups on PND 0[a]	0	0	0.2 ± 0.4	4.0 ± 2.2[**]	
Sex ratio of live pups (male/female)	86/92	72/73	82/93	6/6	
Viability index on PND 4[a,e]	98.9 ± 2.6	99.3 ± 2.1	97.7 ± 3.5	26.7 ± 46.2	
No. of live pups on PND 4[a]	14.7 ± 1.6	14.4 ± 2.1	14.3 ± 2.0	1.3 ± 2.3[**]	
Body weight of live pups on PND 0 (g)[a]					
Male	7.3 ± 0.7	7.4 ± 0.5	7.1 ± 0.6	5.9 ± 0.6	
Female	7.0 ± 0.6	7.0 ± 0.5	6.9 ± 0.6	6.3 ± 0.1	
Body weight of live pups on PND 4 (g)[a]					
Male	11.8 ± 1.0	11.5 ± 0.7	11.0 ± 1.1	9.1	
Female	11.2 ± 1.0	10.9 ± 0.7	10.7 ± 0.9	8.4	
External examination of pups					
No. of pups (litters) examined	178 (12)	145 (10)	176 (12)	28 (4)	
No. of pups (litters) with malformations	0 (0)	0 (0)	0 (0)	1 (1)	
General edema	0 (0)	0 (0)	0 (0)	1 (1)	
Internal examination of pups					
No. of pups (litters) examined	178 (12)	144 (10)	175 (12)	27 (4)	
No. of pups (litters) with malformations	0 (0)	0 (0)	0 (0)	0 (0)	
No. of pups (litters) with variations	8 (6)	3 (2)	18 (7)	1 (1)	
Thymic remnants in the neck	6 (4)	3 (2)	14 (5)	1 (1)	
Left umbilical artery	2 (2)	0 (0)	4 (4)	0 (0)	

[a] Values are given as the mean ± S.D.

[b] Implantation index (%) = no. of implantation sites/no. of corpora lutea × 100.

[c] Delivery index (%) = total no. of pups born/no. of implantation sites × 100.

[d] Live birth index (%) = no. of liver pups on PND 0/total no. of pups born × 100.

[e] Viability index on PND 4 (%) = no. of liver pups on PND 4/no. of live pups on PND 0 × 100.

[*] Significantly different from the control group (P < 0.05).

[**] Significantly different from the control group (P < 0.01).

not ordinarily carnivorous, including nonhuman primates, are nevertheless likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [21].

The malformations and variations found in the current study are those that occur spontaneously among control rats [22–24], and the incidence in the THFA-treated group was very low and not different from that of the control group. However, in the present study, only external and internal examination was performed for pups, and no skeletal examinations were performed. Furthermore, the effects of THFA on the morphological development of offspring could not be evaluated at higher doses because a sufficient number of offspring was not obtained. To accurately evaluate prenatal developmental toxicity, including teratogenicity, it is necessary to interrupt pregnancy a few hours or days before the expected term, either by hysterectomy or the necropsy of maternal animals [21,25]. Such a prenatal developmental toxicity study of THFA is only available as a dose range-finding study using a small number of animals [11]. In this study, an

insufficient number of fetuses were morphologically examined due to high embryonic loss at 500 mg/kg and above. This prenatal study adopted a wide dose range, and the next lowest dose was 100 mg/kg. Prenatal developmental effects of THFA at the higher dose should be examined with a sufficient number of dams and fetuses.

The present study was performed in compliance with the OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [13]. This screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of endpoints, and, therefore, had reduced power in detecting any small effects. Although the results of the current study clearly showed the adverse effects of THFA on the reproduction and development of rats, information on the effects of THFA on reproduction and development is not sufficient at this time. The present results showed that a full reproductive and developmental toxicity study of THFA is required.

In conclusion, the results of this reproductive and developmental toxicity study provide a more comprehensive toxicity profile of THFA than has been previously reported, and the NOAELs for parental and reproductive/developmental toxicity were concluded to be 50 mg/kg/day.

Acknowledgement

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Potential adverse effects of phthalic acid esters on human health: A review of recent studies on reproduction

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Abstract

Various phthalic acid esters (PAEs) have been used for a wide range of products. PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. These findings have raised concern about the possibility of PAEs as contributors to reproductive and developmental adverse effects in humans. This paper focuses on PAE exposure and health effects in human populations and summarizes recent studies. The exposure data in human populations indicate that the current methodology of estimation of PAE exposure is inconsistent. It is therefore important to obtain improved data on human PAE exposure and better understanding of the toxicokinetics of PAEs in each subpopulation. Studies on health effects of PAEs in humans have remained controversial due to limitations of the study designs. Some of findings in human populations are consistent with animal data suggesting that PAEs and their metabolites produce toxic effects in the reproductive system. However, it is not yet possible to conclude whether phthalate exposure is harmful for human reproduction. Studies in human populations reviewed in this paper are useful for showing the strength of the association. It is sometimes claimed that the use of animal data for estimating human risk does not provide strong scientific support. However, because it is difficult to find alternative methods to examine the direct toxic effects of chemicals, animal studies remain necessary for risk assessment of chemicals including PAEs.
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Keywords: Phthalic acid ester; Human health; Reproduction; Development

1. Introduction

Various phthalic acid esters (PAEs) have been used for a wide range of products, and the largest use of these esters is in plasticizers for polyvinyl chloride (PVC) products (Autian, 1973). When used as plasticizers, PAEs are not irreversibly bound to the polymer matrix; therefore, they can migrate from the plastic to the external environment under certain conditions. PAEs are ubiquitous environmental pollutants because of their widespread manufacture, use, and disposal as well as their high concentration in and ability to migrate from plastics (Marx, 1972; Mayer et al., 1972). Humans are exposed to PAEs from food con-

taminated during growth, processing, and packaging or from storage and indoor air. Di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), and butyl benzyl phthalate (BBP) were particularly found in fatty foods including dairy products (Kavlock et al., 2002a,b,c). Women have been exposed to DEHP, DBP, and diethyl phthalate (DEP) in cosmetics on a daily basis (Koo and Lee, 2004).

Some PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. The major toxicities are known to be testicular effects (Zhang et al., 2004), embryolethality (Ema et al., 1994, 1997a; Tyl et al., 1988), malformations such as cleft palate and fusion of the sternebrae, and adverse effects on sexual differentiation (Ema et al., 1997b, 1998; Gray et al., 2000). There are considerable homologies among different

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mammalian species for androgen activities during sex differentiation (Gray et al., 1994). Chemicals that adversely affect human sex differentiation (Schardein, 2000) also produce predictable alterations of this process in rodents (Gray et al., 1994). The anti-androgenic effects of some PAEs were observed in a Hershberger assay in castrated male rats (Stroheker et al., 2005; Lee and Koo, 2007) or in an AR reporter gene assay (Satoh et al., 2004). These findings have raised concern about the possibility of PAEs as contributors to reproductive and developmental adverse effects in humans. Available data on primates are currently limited but show significant differences from rodents regarding the reproductive effects of PAEs (Kurata et al., 1998; Pugh et al., 2000; Tomonari et al., 2006), indicating the possibility of species-related differences.

The lower sensitivity of primates is thought to arise from differences between rodents and primates in the absorption, distribution, metabolism, and excretion (ADME) of PAEs. Monoester metabolites of PAEs such as mono-2-ethylhexyl phthalate (MEHP) and mono-butyl phthalate (MBP) have been reported to be the active metabolites responsible for adverse effects (Elcombe and Mitchell, 1986; Ema and Miyawaki, 2001; Tomita et al., 1986). DEHP is hydrolyzed to MEHP by the catalytic action of lipase (Ito et al., 2005). Lipase activities in the liver, small intestine, and kidneys are higher in rodents than in primates (Ito et al., 2005). The maximum concentrations of MEHP in the blood of marmosets were up to 7.5 times lower than in rats (Kessler et al., 2004). In rats, MEHP is oxidized to other secondary metabolites, and both MEHP and secondary metabolites are found in the blood and amniotic fluid primarily in their free form (Kurata et al., 2005; Calafat et al., 2006). Urinary MEHP was mostly found as a glucuronide conjugate in rats (Calafat et al., 2006). On the other hand, in humans and primates, MEHP is present in blood and urine primarily as glucuronide conjugates, which enhance urinary excretion and reduce the biological activity of the active metabolites (Ito et al., 2005; Kurata et al., 2005; Silva et al., 2003), but DEHP metabolites with a carboxylated ester side-chain were found as both conjugates and free forms in human urine (Silva et al., 2006a). Plasma radioactivity measurements of DEHP in rats and marmosets revealed that radioactivity in rat testis was about 20-fold higher than that in marmosets. About 60% of the dose was excreted in urine in rats primarily as unconjugated MEHP-metabolites. For marmosets, the majority of the dose was excreted in the feces (Kurata et al., 1998).

The potential of PAEs to produce adverse effects in humans has been the subject of considerable discussion. Many toxicity studies have been conducted in laboratory animals, especially in rats, and review papers are available based on these animal data (Corton and Lapinskas, 2005; Ema, 2002; Foster, 2006); however, studies in human populations have not been adequate to assess the toxic potential on human health. Lately, several review papers were published regarding PAE exposure in human populations (Koch et al., 2006; Latini, 2005; Schettler, 2006). These review studies are worthwhile for knowing exposure levels and routes of PAE exposure in human populations; however, review works regarding the relationships between PAE exposure and human health are not adequate. In the late 20th century, only a few papers have reported a relationship between environmental PAE exposures and human health (Aldyreva et al., 1975; Fredricsson et al., 1993; Murature et al., 1987). Studies in human populations have been receiving much attention for the last 2 or 3 years, and the number of studies in human populations has increased. Some recent studies have suggested possible associations between environmental exposure to PAEs and adverse effects on human reproductive health. It will be useful to review them to determine whether there is concordance between animal models and human populations in order to develop hypotheses for future studies. This paper focuses on the PAE exposure and health effects in human populations and summarizes recent human studies published up to 2006.

2. Exposure to PAEs

Many studies have suggested that PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. Although the most of these animals were exposed to PAEs at relatively high level to exam toxicological effects, some studies showed that relatively low doses of PAEs caused toxic effects (Arcadi et al., 1998; Lee et al., 2004; Poon et al., 1997). Thus, there is a question of whether humans are exposed to PAEs at a severe enough level to generate human health effects. Several studies have been conducted to estimate the exposure level of PAEs in humans.

2.1. Estimate of PAE exposure in human populations

Levels of human exposure to PAEs were estimated from the urinary metabolite of PAEs. Table 1 shows the urinary PAE metabolite in US populations. A pilot study was conducted for measurement of levels of seven urinary phthalate metabolites, MEHP, MBP, mono-benzyl phthalate

Table 1

Total urinary phthalate monoester concentrations (in µg/g of creatinine)

Metabolites	Diester	Measurement in 289 individuals (Blount et al., 2000)		Measurement in 2541 individuals (Silva et al., 2004a)	
		Geometric mean	95th percentile	Geometric mean	95th percentile
MEP	DEP	345	2610	163	1950
MBP	DBP/BBP	36.9	162	22.4	97.5
MBzP	BBP	20.2	91.9	14.0	77.4
McHP	DcHP	0.3	1.0	<LOD	3.00
MEHP	DEHP	3.0	15.2	3.12	18.5
MOP	DOP	0.5	2.1	<LOD	3.51
MINP	DINP	1.3	6.8	<LOD	4.29

LOD, limit of detection.

(MBzP), mono-cyclohexyl phthalate (McHP), mono-ethyl phthalate (MEP), mono-isononyl phthalate (MINP) and mono-*n*-octyl phthalate (MOP), in 289 US adults (Blount et al., 2000). A subsequent study involving a group of 2541 individuals from participants of the National Health and Nutrition Examination Survey (NHANES) aged ⩾ 6 years in US provided similar findings to the previous study although urinary levels for MEP, MBP and MBzP were lower than the previously reported values (Silva et al., 2004a).

These urinary metabolite levels were used to calculate the ambient exposure levels for five PAEs, BBP, DBP, DEHP, di-*n*-octyl phthalate (DOP) and di-isononyl phthalate (DINP), in human populations (David et al., 2001; Kohn et al., 2000). The estimation of daily intake of phthalates was calculated by applying the following equation according to David et al. (2001):

$$\text{Intake } (\mu g/kg/day) = \frac{UE \ (\mu g/g) \times CE \ (mg/kg/day)}{f \times 1000 \ (mg/g)}$$
$$\times \frac{MW_d}{MW_m}$$

where UE is the urinary concentration of monoester per gram creatinine, CE is the creatinine excretion rate normalized by body weight, f is the ratio of urinary excretion to total elimination, and MW_d and MW_m are the molecular weights of the diesters and monoesters, respectively.

Table 2 shows the estimated ambient exposure to PAEs. As shown in Table 2, all estimated PAE intakes in the US population were lower than the tolerable daily intake (TDI) values settled by the EU Scientific Committee for Toxicity, Ecotoxicity and the Environment (BBP: 200 μg/kg/day, DBP: 100 μg/kg/day, DEHP: 37 μg/kg/day, DOP: 370 μg/kg/day, and DINP:150 μg/kg/day) (CSTEE, 1998), the reference dose (RfD) of the US EPA (BBP: 200 μg/kg/day, DBP: 100 μg/kg/day, and DEHP: 20 μg/kg/day) (US EPA, 2006) and the TDI values established by the Japanese Government (DEHP: 40–140 μg/kg/day and DINP: 150 μg/kg/day) (MHLW, 2002). Among these PAEs, DEHP is most commonly used plasticizer for flexible PVC formulations and is a widespread environmental contaminant (Kavlock et al., 2002c); however, the

estimated daily intake level of DEHP was not high as expected.

Koch et al. (2004a, 2003) and Barr et al. (2003) cast doubt on the sensitivity of the biomarker MEHP for assessing DEHP exposure, and they explored mono- (2-ethyl-5oxo-hexyl) phthalate (5oxo-MEHP) and mono- (2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) as additional biomarkers for DEHP. After a single oral dose of DEHP in a male volunteer, peak concentrations of MEHP, 5OH-MEHP, and 5oxo-MEHP were found in the serum after 2 h, and in urine after 2 h (MEHP) and 4 h (5OH-MEHP and 5oxo-MEHP). The major metabolite was MEHP in serum and 5OH-MEHP in urine (Koch et al., 2004a). Barr et al. (2003) analyzed 62 urine samples for metabolites of DEHP, and the mean urinary levels of 5oxo-MEHP and 5OH-MEHP were 4-fold higher than MEHP.

Koch et al. (2003) determined a median DEHP intake of 13.8 μg/kg/day based on urinary oxidative metabolites of DEHP, 5OH-MEHP and 5oxo-MEHP, in male and female Germans (*n* = 85; aged 18–40). Twelve percent of the subjects exceeded the TDI of the EU-CSTEE (37 μg/kg/day) and 31% of the subjects exceeded the RfD of the US EPA (20 μg/kg/day). For DBP, BBP, DEP, and DOP, the 95th percentile intake values were estimated to be 16.2, 2.5, 22.1, and 0.42 μg/kg/day, respectively. Subsequently, urine samples from 254 German children aged 3–14 were also analyzed for concentrations of these three metabolites of DEHP. The geometric means for MEHP, 5OH-MEHP and 5oxo-MEHP in urine were 7.9, 52.1, and 39.9 μg/L, respectively (Becker et al., 2004). The median daily intake of DEHP in children was estimated to be 7.7 μg/kg. Four children exceeded the TDI of the EU-CSTEE (37 μg/kg/day) and 26 children also exceeded the RfD of the US EPA (20 μg/kg/day) (Koch et al., 2006).

Although these findings showed that German populations could be exposed to DEHP at a higher level than previously estimated values (David et al., 2001; Kohn et al., 2000), these results should be interpreted carefully. In the above-mentioned equation, Kohn et al. (2000) and David et al. (2001) applied the fractional urinary excretion value (f = 0.106: MEHP) determined by Peck and Albro (1982). On the other hand, Koch et al. (2003) applied the fractional urinary excretion values (f = 0.074: 5OH-

Table 2
Comparison of calculated intakes of phthalates based on the geometric mean values for urinary metabolites and the tolerable daily intake levels as well as the reference dose of phthalates (in μg/kg/day)

PAEs	Estimated by David et al. (2001) for 289 US individuals (Blount et al., 2000)		Estimated by Kohn et al. (2000) for 2541 US individuals (Silva et al., 2004a)		TDI (EU) (CSTEE, 1998)	RfD (US) (US EPA, 2006)	TDI (Japan) (MHLW, 2002)
	Geometric mean	95th percentile	Geometric mean	95th percentile			
BBP	0.73	3.34	0.88	4.0	200	200	Not established
DBP	1.56	6.87	1.5	7.2	100	100	Not established
DEHP	0.60	3.05	0.71	3.6	37	20	40–140
DOP	<LOD	—	0.0096	0.96	370	Not established	Not established
DINP	0.21	1.08	<LOD	1.7	150	Not established	150

LOD, limit of detection.

MEHP, 0.055: 5oxo-MEHP and 0.024: MEHP) determined by Schmid and Schlatter (1985). Using different fractional urinary excretion values can yield several fold differences in estimated values even if the levels of the urinary metabolites are the same.

Table 3 shows a comparison of the estimated median exposure levels of DEHP. Koo and Lee (2005) and Fujimaki et al. (2006) applied the same fractional urinary excretion values of Koch et al. (2003) for calculating daily DEHP intake. Koo and Lee (2005) estimated daily intake of DEHP in Korean children aged 11–12 years old ($n = 150$) and in Korean women aged 20–73 years old ($n = 150$) with a fractional urinary excretion value of 0.024 for MEHP. Median intake levels of DEHP were estimated to be 6.0 μg/kg/day in children and 21.4 μg/kg/day in adult women. TDI of the EU (37 μg/kg/day) was reached at the 56th percentile for women and the 95th percentile for children. Fujimaki et al. (2006) estimated the daily intake of DEHP in forty pregnant Japanese women. The median concentrations of MEHP, 5OH-MEHP and 5oxo-MEHP in the urine were 9.83, 10.4, and 10.9 μg/L, respectively. The median DEHP intake based on MEHP, 5OH-MEHP, and 5oxo-MEHP were estimated to be 10.4 (3.45–41.6), 4.55 (0.66–17.9), and 3.51 (1.47–8.57) μg/kg/day, respectively. These two studies showed higher exposure levels than the previously estimated values in the US population (David et al., 2001; Kohn et al., 2000). Koo and Lee (2005) also showed that a different estimation model can yield 10-fold lower values when estimating DEHP intake, indicating that methods for estimation of daily intake values of PAEs remain inconsistent.

Recently, other secondary oxidized metabolites of DEHP have been recognized (Koch et al., 2005b). Although 5OH-MEHP and 5oxo-MEHP in the urine reflect short-term exposure levels of DEHP, other secondary oxidized metabolites of DEHP such as mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP) and mono-[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) are considered excellent parameters for measurement of the time-weighted body burden of DEHP due to their long half-times of elimination. Biological monitoring in a German population ($n = 19$) indicated that 5cx-MEPP is the major urinary

metabolite of DEHP. Median concentrations of the metabolites of DEHP were 85.5 μg/L (5cx-MEPP), 47.5 μg/L (5OH-MEHP), 39.7 μg/L (5oxo-MEHP), 9.8 μg/L (MEHP) and 36.6 μg/L (2cx-MMHP) (Preuss et al., 2005). Furthermore, oxidized metabolites of DINP have been recently introduced as new biomarkers for measurement of DINP exposure (Koch and Angerer, 2007; Silva et al., 2006b). These new findings imply that more accurate methods for estimation of PAE exposure can be developed.

2.2. Exposure in fetuses and infants

PAE exposure to the fetus in utero is a great concern because some PAEs are considered to be developmental toxicants. Adibi et al. (2003) measured of urinary phthalate metabolites in pregnant women ($n = 26$) in New York. The median creatinine-adjusted concentrations of MEP, MBP, MBzP, and MEHP were 236, 42.6, 12.1, and 4.06 μg/g, respectively. Metabolites levels in pregnant women were comparable with those in US general population (Blount et al., 2000; Silva et al., 2004a). Another study in 24 mother–infant pairs confirmed DEHP and/or MEHP exposure during human pregnancies (Latini et al., 2003a). The mean DEHP concentrations in maternal plasma and cord plasma were 1.15 and 2.05 μg/mL, respectively, and the mean MEHP concentration was 0.68 μg/mL in both maternal plasma and cord plasma. The levels of phthalate metabolites in the amniotic fluid may reflect fetal exposure to PAEs. Only three metabolites, MEP, MBP, and/or MEHP, were detected in the amniotic fluid samples ($n = 54$). The levels of mono-methylphthalate (MMP), MBzP, McHP, MINP, MOP, 5OH-MEHP, and 5oxo-MEHP were under the limits of detection. Levels of MEP, MBP, and MEHP ranged from under the limits of detection to 9.0 ng/mL ($n = 13$), 263.9 ng/mL ($n = 50$), and 2.8 ng/mL ($n = 21$), respectively (Silva et al., 2004b). These studies suggest that human exposure to PAEs can begin in utero.

Breast milk and infant formula can be routes of PAE exposure for infants. Table 4 shows phthalate monoesters levels in human milk, infant formula, and consumer milk. Levels of phthalate monoesters in pooled breast milk

Table 3
Comparison of estimated mean daily intake of DEHP (μg/kg/day)

Metabolites	German[a] adults ($n = 85$) (Koch et al., 2003)	Korean[a] (Koo and Lee, 2005)		Japanese[a] pregnant women ($n = 40$) (Fujimaki et al., 2006)	US[b] adults ($n = 289$) (David et al., 2001)	US[b] aged ≥6 years ($n = 2541$) (Kohn et al., 2000)
		Adults (women) ($n = 150$)	Children ($n = 150$)			
MEHP	10.3 (38.3)	21.4 (158.4)	6.0 (37.2)	10.4	0.60 (3.05)	0.71 (3.6)
5OH-MEHP	13.5 (51.4)	No data	No data	4.55	No data	No data
5oxo-MEHP	14.2 (52.8)	No data	No data	3.51	No data	No data
Oxidative DEHP metabolites[c]	13.8 (52.1)	No data	No data	No data	No data	No data

Figures in parentheses show the 95th percentile.
[a] Applying the equation of David et al. (2001) and the fractional urinary excretion value determined by Schmid and Schlatter (1985).
[b] Applying the equation of David et al. (2001) and the fractional urinary excretion value determined by Peck and Albro (1982).
[c] Average of estimated intakes of DEHP based on 5OH-MEHP and 5oxo-MEHP.

Table 4
Phthalate monoester levels (µg/L) in human milk, infant formula and consumer milk

Monoester	Diester	Three pooled breast milk samples (Calafat et al., 2004b)	Thirty-six samples of Danish mother's milk (Mortensen et al., 2005)	Ten samples of infant formula (Mortensen et al., 2005)	Seven samples of consumer milk (Mortensen et al., 2005)
MMP	DMP	<LOD	0.17 ± 0.26[a]	<LOD	<LOD
MEP	DEP	<LOD	1.78 ± 2.74	<LOD	<LOD
MBP	DBP/BBP	1.3 ± 1.5[a]	359 ± 1830	0.6–3.9[b]	1.4–2.8[b]
MBzP	BBP	<LOD	1.2 ± 1.6	<LOD	<LOD
MEHP	DEHP	7.8 ± 6.8	13 ± 11	5.6–9.1	7.1–9.9
MINP	DINP	15.9 ± 7.7	114 ± 69	<LOD	<LOD

LOD, limit of detection.
[a] Values are given as mean ± standard deviation.
[b] Values are given as range.

($n = 3$) were reported by Calafat et al. (2004b). A subsequent study for 36 individual human milk samples provided higher values for all metabolites; in particular, levels of MBP were two magnitudes higher (Mortensen et al., 2005) than that in the previous study by Calafat et al. (2004b). Phthalate metabolites in breast milk were detected in their free forms unlike the metabolites found in urine and blood. Therefore, infants may receive active PAE metabolites from breast milk on a daily basis. Only MBP and MEHP were detected in consumer milk and infant formula (Mortensen et al., 2005).

The levels of PAEs were determined for 27 infant formulae sold in several countries, and DEHP and DBP were found (Yano et al., 2005). The amounts of DEHP (34–281 ng/g) were much higher than DBP (15–77 ng/g). DEHP, DBP, and DEP were also found in a total of 86 human milk samples collected from 21 Canadian mothers over a 6-month postpartum period. DEHP was the major ester with a mean value of 222 ng/g (8–2920 ng/g), followed by DBP with a mean of 0.87 ng/g (undetectable to 11.39 ng/g). DEP with a mean of 0.31 ng/g (undetectable to 8.1 ng/g) was detected in only a small number of samples. Dimethyl phthalate (DMP), BBP, and DOP were not detected in any samples (Zhu et al., 2006). Table 5 presents estimated maximum daily intakes of PAEs in infants, which was calculated by assuming that the body weight of infants is 7 kg and the daily intake of milk is 700 mL. Although the total estimated maximum daily intake of DEHP in infants was generally less than in general adults (Koch et al., 2003), the estimated maximum daily intake per body weight was higher than adults due to the low body weight of the infants. Assuming that milk was the only exposure route for PAEs in the infants, it is likely that infants had less exposure to DBP and DEP than the general adult population (Koch et al., 2003). These studies suggest that some infants may also be exposed to DEHP at higher levels than the established safe standard levels.

2.3. Possible variation of PAE exposure

Some humans may be exposed to PAEs at higher level than the established safe standard levels. Measurements of urinary metabolites of PAEs have revealed notable differences in concentrations of specific metabolites based on age, gender and race (Blount et al., 2000; Silva et al., 2004a). Concentrations of MBP, MBzP, and MEHP were higher in the youngest age group (6–11 years) and decreased with age. Non-Hispanic blacks tended to have higher levels of phthalate metabolites than non-Hispanic whites or Mexican Americans. Females tended to have a higher level of phthalate metabolites than did males (Silva et al., 2004a). Blount et al. (2000) also indicated that women of reproductive age (20–40 years) had significantly higher levels of MBP than other age/gender groups. Measurement of the three urinary metabolites MEHP, 5OH-MEHP and 5oxo-MEHP in male and female children ($n = 254$) aged 3 to 14 showed that boys had higher concentrations of these three metabolites of DEHP than girls (Becker et al., 2004). The higher levels of PAE metabolites in the young age group may be due to a different food category, dairy products, or the use of PVC toys (CSTEE, 1998), and the higher levels of MBP in females may be

Table 5
Estimated maximum daily intake (µg/kg/day) of PAEs in infants and general German population

Compounds	Human milk ($n = 21$) (Zhu et al., 2006)	Infant formula ($n = 27$) (Yano et al., 2005)	General population ($n = 85$) (Koch et al., 2003)
DEHP	301 (41.1)	6.9	166 (52.1)
DBP	1.21 (0.12)	1.07	22.6 (16.2)
DEP	0.87	Not measured	69.3 (22.1)

Daily PAE intake levels were calculated by assuming that the average daily milk consumption is 700 mL (722 g: specific gravity of human milk = 1.031) and average body weight is 7 kg.
Figures in parentheses show 95th percentile.

due to use of cosmetic products that contain high levels of DBP (Koo and Lee, 2004).

Koo et al. (2002) approached this issue from a different point of view. Their statistical examination concluded that higher levels of MBP in urine were associated with a lower level of education (only a high school education) and/or lower family income (less than $1500) in the month before sampling. Slightly higher levels of MEP were found in urban populations, low income groups, and males. PAE exposure occurred from food, water, and indoor air, although dietary intake of PAEs from contaminated food was likely to be the largest source (Schettler, 2006). Education level and family income may therefore influence the dietary pattern.

It is still unknown whether the variations in these metabolites represent differences in the actual exposure levels. Metabolism of PAEs may vary by age, race, or sex; for example, the ratios of 5OH-MEHP/5oxo-MEHP and 5oxo-MEHP/MEHP decrease with increasing age (Becker et al., 2004). The mean relative ratios of urinary MEHP to 5OH-MEHP to 5oxo-MEHP were 1 to 7.1 to 4.9 in German male and female children and 1 to 3.4 to 2.1 in German male and female adults. This might indicate enhanced oxidative metabolism in children (Koch et al., 2004b). The ratios for urinary MEHP, 5OH-MEHP and 5oxo-MEHP in Japanese pregnant women were reported to be approximately 1 to 1 to 1 (Fujimaki et al., 2006). The variation seen in these three populations may be due to differences in the analytical methods; however, these variations in human populations are still not negligible for accurate risk assessment. Because the current estimates of PAE intake in humans can be imprecise and ADMEs of PAEs in each subpopulation are not clear, the significance of exposure to PAEs with regard to health effects is yet unknown.

2.4. Exposure from medical devices

DEHP has been used for a wide variety of PVC medical devices such as i.v. storage bags, blood storage bags, tubing sets, and neonatal intensive care units (NICUs), and known treatments that involve high DEHP exposures include blood exchange transfusions, extracorporeal membrane oxygenation and cardiovascular surgery.

Serum concentrations of DEHP were significantly increased in platelet donors and receptors (Buchta et al., 2005, 2003; Koch et al., 2005c). A median increase of 232% of serum DEHP was detected after plateletpheresis in healthy platelet donors (Buchta et al., 2003). Mean DEHP doses for discontinuous-flow platelet donors and continuous-flow platelet donors were 18.1 and 32.3 µg/kg/day on the day of apheresis, which were close to or exceeded health standard levels such as the TDI or RfD (Koch et al., 2005c).

Premature infants who experience medical procedures may have a higher risk of exposure to DEHP than the general population. Because the same size of each medical device is used for all ages, infants may receive a larger dose

of PAEs on a mg/kg basis than adults due to their smaller size. Calafat et al. (2004a) provided the first quantitative evidence confirming that infants who undergo intensive therapeutic medical interventions are exposed to higher concentrations of DEHP than the general population. They assessed exposure levels of DEHP in 6 premature newborns (23–26 weeks old) by measuring levels of urinary MEHP, 5OH-MEHP and 5oxo-MEHP. The geometric mean concentrations of MEHP (100 µg/L), 5oxo-MEHP (1617 µg/L), and 5OH-MEHP (2003 µg/L) were found to be one or two orders of magnitude higher than German children aged 3–5 (MEHP: 6.96 µg/L, 5OH-MEHP: 56.7 µg/L and 5oxo-MEHP:42.8 µg/L). Koch et al. (2005a) estimated DEHP exposure due to medical devices by using five major DEHP metabolites. Forty-five premature neonates (2–31 days old) with a gestational age of 25–40 weeks at birth were exposed to DEHP up to 100 times over the RfD value set by the US EPA depending on the intensity of medical care (median: 42 µg/kg/day; 95th percentile: 1780 µg/kg/day).

3. Health effects of PAEs in human populations

In the late 20th century, a few studies reported a relationship between environmental exposure of PAEs and human health. For example, Murature et al. (1987) reported that there was a negative correlation between DBP concentration in the cellular fraction of ejaculates and sperm production. Fredricsson et al. (1993) reported that human sperm motility was affected by DEHP and DBP. In females, decreased rates of pregnancy and higher levels of miscarriage in factory workers were associated with occupational exposure of DBP (Aldyreva et al., 1975). More recent studies in human males, females and infants are summarized below.

3.1. Studies of the male reproductive system

Table 6 shows a summary of studies of the male reproductive system in human populations. Two studies are available for 168 male subjects who were members of subfertile couples (Duty et al., 2003a,b). Eight urinary PAE metabolites, MEP, mono-methyl phthalate (MMP), MEHP, MBP, MBzP, MOP, MINP and McHP, were measured with a single spot urine sample. Urinary MEHP, MOP, MINP, or McHP showed no relevance to sperm parameters or DNA damage (Duty et al., 2003a,b). Urinary MBP was associated with lower sperm concentration and lower motility, and urinary MBzP was associated with lower sperm concentration. There was limited evidence suggesting an association of increased MMP with poor sperm morphology (Duty et al., 2003a). A neutral comet assay revealed that urinary MEP levels were associated with increased DNA damage in sperm (Duty et al., 2003b). This result was confirmed by a recent study in 379 men from an infertility clinic in which sperm DNA damage was associated with MEP (Hauser et al., 2007).

Table 6
Male reproductive effects in human populations

Compounds	Number of subjects	Related effects	Reference
Total PAEs[a]	$n = 21$	↓Sperm normal morphology, ↑Percent of single-stranded DNA in sperm	Rozati et al. (2002)
Phthalic acid	$n = 234$	↑Large testis[c], ↑Sperm motility[c]	Jonsson et al. (2005)
DEHP	$n = 37$	↓Semen volume, ↑Rate of sperm malformation	Zhang et al. (2006)
MEHP	$n = 187$	↓Straight-line velocity and curvilinear velocity of sperm[d]	Duty et al. (2004)
	$n = 74$	↓Plasma free testosterone	Pan et al. (2006)
%MEHP[b]	$n = 379$	↑Sperm DNA damage	Hauser et al. (2007)
MEP	$n = 168$	↑DNA damage in sperm	Duty et al. (2003b)
	$n = 234$	↑Large testis[c], ↓Sperm motility, ↓Luteinizing hormone	Jonsson et al. (2005)
	$n = 379$	↑DNA damage in sperm	Hauser et al. (2007)
	$n = 187$	↓Sperm linearity[d], ↑Straight-line velocity and curvilinear velocity of sperm[c,d]	Duty et al. (2004)
DBP	$n = 37$	↓Semen volume	Zhang et al. (2006)
MBP	$n = 168$	↓Sperm concentration, ↓Sperm motility	Duty et al. (2003a)
	$n = 463$	↓Sperm concentration, ↓Sperm motility	Hauser et al. (2006)
	$n = 187$	↓Straight-line velocity and curvilinear velocity of sperm[d]	Duty et al. (2004)
	$n = 74$	↓Plasma free testosterone	Pan et al. (2006)
	$n = 295$	↑Inhibin B level[c,d]	Duty et al. (2005)
MBzP	$n = 168$	↓Sperm concentration	Duty et al. (2003a)
	$n = 463$	↓Sperm concentration[d]	Hauser et al. (2006)
	$n = 187$	↓Straight-line velocity and curvilinear velocity of sperm[d]	Duty et al. (2004)
	$n = 295$	↓Follicle-stimulating hormone[c]	Duty et al. (2005)
MMP	$n = 168$	↑Poor sperm morphology[d]	Duty et al. (2003a)

[a] Total level of DMP, DEP, DBP, DEHP and DOP.
[b] The urinary concentrations of MEHP divided by sum of MEHP, 5OH-MEHP and 5oxo-MEHP concentrations and multiplied by 100.
[c] Data do not support the association of PAEs with reproductive adverse effects in male human populations.
[d] Only suggestive association was observed (statistically not significant).

In another study, semen volume, sperm concentration, motility, sperm chromatin integrity and biochemical markers of epididymal and prostatic function were analyzed together with MEP, MEHP, MBzP, MBP, and phthalic acid levels in urine in 234 young Swedish men (Jonsson et al., 2005). Urinary MEP level was associated with fewer motile sperm, more immotile sperm, and lower serum luteinizing hormone (LH) values. However, higher phthalic acid levels were associated with more motile sperm and fewer immotile sperm; therefore, the results for phthalic acid were opposite what had been expected.

A similar study was conducted in 463 male partners of subfertile couples (Hauser et al., 2006). Phthalate metabolites were measured in a single spot urine sample. There were dose–response relationships of MBP with low sperm concentration and motility. There was suggestive evidence of an association between the highest MBzP quartile and low sperm concentration. There were no relationships between MEP, MMP, MEHP or oxidative DEHP metabolites with any of the semen parameters.

Although there were associations between some metabolites of PAEs and sperm count, motility, or morphology, no statistically significant associations between MEP, MBzP, MBP, MEHP, or MMP and sperm progression, sperm vigor, or swimming pattern were observed in 187 subjects. There were only suggestive associations as follows: negative associations between MBzP with straight-line velocity (VSL) or curvilinear velocity (VCL), between MBP with VSL and VCL and between MEHP with VSL and VCL. MEP was positively associated with VSL and VCL but negatively associated with linearity (Duty et al., 2004).

Duty et al. (2005) explored the relationship between urinary phthalate monoester concentrations and serum levels of reproductive hormones in 295 men. In their previous studies (Duty et al., 2003a,b), MBP and MBzP were associated with sperm parameters, and the investigators had hypothesized that inhibin B, a sensitive marker of impaired spermatogenesis (Uhler et al., 2003), would be inversely associated with MBP and MBzP. However, MBP exposure was associated with increased inhibin B, although this was of borderline significance. Additionally, MBzP exposure was significantly associated with a decrease in serum follicle-stimulating hormone (FSH) level. The serum FSH level has been used as a marker of spermatogenesis for infertile males in clinical evaluation (Subhan et al., 1995), and it is increased in comparison to normal males (Sina et al., 1975). Therefore, the hormone concentrations did not change in the expected patterns.

DEHP is known to cause adverse effects on the male reproductive system in rodents (Gray et al., 2000), and DNA damage in human lymphocytes is also induced by DEHP and MEHP (Anderson et al., 1999). A Hershberger assay with DEHP or MEHP showed anti-androgenic effects in castrated rats (Stroheker et al., 2005; Lee and Koo, 2007). However, only a few studies have suggested that DEHP could be a reproductive toxicant in humans. Urine and blood samples from 74 male workers at a factory producing unfoamed polyvinyl chloride flooring exposed to DBP and DEHP were compared with samples from 63 unexposed male workers. The exposed workers had significantly elevated concentrations of MBP (644.3 vs. 129.6 μg/g creatinine) and MEHP (565.7 vs. 5.7 μg/g

creatinine) in their urine. The plasma free testosterone level was significantly lower (8.4 vs. 9.7 µg/g creatinine) in the exposed workers than in the unexposed workers. Free testosterone was negatively correlated to MBP and MEHP in the exposed worker group (Pan et al., 2006). Another recent study showed that although the urinary MEHP concentration was not associated with sperm DNA damage, the percentage of DEHP metabolites excreted as MEHP (MEHP%) was associated with increased sperm DNA damage. It is of interest that the oxidative metabolites had inverse relationships with sperm DNA damage (Hauser et al., 2007).

Unlike other studies, the following two studies used diester concentrations for measurement of PAEs. Rozati et al. (2002) reported that the concentration of total PAEs (DMP, DEP, DBP, BBP, DEHP, and DOP) in the seminal plasma was significantly higher in infertile men ($n = 21$) ,compared to controls ($n = 32$). Correlations were observed between seminal PAEs and sperm normal morphology ($r = -0.769$, $p < .001$), in addition to the % of single-stranded DNA in the sperm ($r = 0.855$, $p < .001$). This study examined only total PAEs, and relationships between individual PAEs and sperm parameters were not identified. Another study in a human male population was carried out by measurement of semen parameters and DEHP, DBP, and DEP in human semen ($n = 37$) (Zhang et al., 2006). The three PAEs were detected in most of the samples, and mean levels of DEHP, DBP, and DEP were 0.28, 0.16, and 0.47 µg/L, respectively. There was a negative correlation between semen volume and concentration of DBP or DEHP. There was also a positive association between the rate of sperm malformation and DEHP concentrations. These diester concentrations may directly reflect PAE exposure levels.

Animal data have suggested that mature exposure to DBP and DEHP affects sperm parameters (Agarwal et al., 1986; Higuchi et al., 2003). Dietary exposure of mature male F344 rats (15–16 weeks old) to DEHP (0–20,000 ppm) for 60 consecutive days resulted in a dose dependent reduction in testis, epididymis and prostate weights at 5000 and 20,000 ppm (284.1 and 1156.4 mg/kg/day). Epididymal sperm density and motility were also reduced and there was an increased occurrence of abnormal sperm at 20,000 ppm (Agarwal et al., 1986). Exposure of BBP from adolescence to adulthood showed changes in reproductive hormones in CD(SD)IGS rats at 100 and 500 mg/kg/day (Nagao et al., 2000). In Dutch-Belted rabbits, exposure of DBP during adolescence and in adulthood decreased the amount of normal sperm whereas in utero exposure of DBP decreased the amount of normal sperm, sperm counts, ejaculated volume, and accessory gland weight (Higuchi et al., 2003). Preadolescent male rats appear to have a greater sensitivity to the adverse testicular effects of DEHP than older rats. Akingbemi et al. (2001) demonstrated that preadolescent male rats (21 days old) were more sensitive than young adult animals (62 days old) to 14- or 28-day DEHP exposures that

induced decreases in Leydig cell production of testosterone. PAE effects on male reproductive organs could be influenced by the stage of development, but the data also support the possibility that mature animals are susceptible to PAE exposure. The studies in human populations were in accord with these animal data.

Some studies in human populations have suggested associations between MEP, a metabolite of DEP, and changes in sperm; however, these results regarding to MEP are not supported by animal studies. According to Foster et al. (1980), oral dosing of DEP (1600 mg/kg/day) for 4 days did not damage the testes in young SD rats. In another study, male and female CD-1 mice were given diets with DEP (0–2.5%) for 7 days prior to and during a 98-day cohabitation period. There were no apparent effects on reproductive function in animals exposed to DEP (Lamb et al., 1987).

Furthermore, studies in rodents may have little relevance to humans for the reason that DEHP and DINP do not cause reproductive effects in non-human primates. Pugh et al. (2000) showed no evidence of testicular lesions in young adult cynomolgus monkeys (~2 years old) gavage dosed with 500 mg/kg bw/day DEHP and DINP for 14 days. A study with matured marmosets (12–15 months old) showed that repeated dosing of DEHP at up to 2500 mg/kg bw/day for 13 weeks resulted in no differences in testicular weight, prostate weight, blood testosterone levels, blood estradiol levels or any other aspect of the reproductive system (Kurata et al., 1998). DEHP treatment up to 2500 mg/kg bw/day in marmosets from weaning (3 months old) to sexual maturation (18 months old) produced no evidence of testicular damage. Sperm head counts, zinc levels, glutathione levels and testicular enzyme activities were also not affected (Tomonari et al., 2006). In contrast to data from rabbits and rodents, no testicular effects of DEHP or DINP were found in non-human primates at any ages. The current understanding of how PAEs affects semen parameters, sperm DNA damage, and hormones in human populations is limited and further investigation is required.

3.2. Studies of the female reproductive system

Studies of adult female humans are less numerous than those of adult males. Cobellis et al. (2003) compared plasma concentrations of DEHP and MEHP in endometriotic women ($n = 55$) with control women ($n = 24$), and higher plasma DEHP concentrations were observed in endometriotic women. Similar results were observed in a recent study reported by Reddy et al. (2006). The investigators collected blood samples from 49 infertile women with endometriosis (the study group), 38 infertile women without endometriosis (control group I) and 21 women with proven fertility (control group II). Women with endometriosis showed significantly higher concentrations of DBP, BBP, DOP, and DEHP when compared to both control groups. Upon analysis of cord blood samples of 84 newborns, Latini et al. (2003b) revealed that MEHP-positive