Effects of di-iso-butyl phthalate on testes of prepubertal rats and mice

By

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Summary: Di-iso-butyl phthalate (DiBP), a special plasticizer, is used as a substitute for di(n-butyl) phthalate (DBP). The effects of DiBP on testes in prepubertal rodents still remain to be obscure. Testicular toxicity of DiBP was investigated in 21-day-old Sprague-Dawley rats and C57BL/6N mice, using with *in situ* TUNEL method. For an acute exposure experiment, animals were once given DiBP at various concentrations by oral gavage. For a subchronic exposure experiment, they were daily given DiBP at various concentrations for consecutive 7 days. Controls were treated with corn oil under the same condition. For a recovery experiment, rats were once given DiBP (1000 mg/kg), and were sacrificed at day 1 to 8 after administration. Furthermore, the disorder of vimentin filaments in Sertoli cells after daily administration of DiBP (500 mg/kg) for consecutive 7 days in rats also identified by immunohistochemistry using anti-vimentin antibody. As a result, the present study demonstrated that DiBP can induce testicular atrophy in rats due to the increase of TUNEL-positive spermatogenic cells in both acute and subchronic exposure experiments. At the same time, the disorder of vimentin filaments in Sertoli cells was recognized. However, no such damages could be found in mouse testis. For the recovery experiment, the testis weight and testicular morphology returned to normal at day 6 after administration. In conclusion, the present study indicates that DiBP causes the significant increase of TUNEL-positive spermatogenic cells and the disorder of vimentin filaments in Sertoli cells in rats and that DiBP shows a species-specific toxicity.

Introduction

Phthalate esters, widely used as a plasticizer in the manufacturing process of consumer products, are known to impair spermatogenesis at postnatal stage and disturb the development of male reproductive system at fetal stage in rodents. For example, administration of di-butyl phthalate (DBP) to male rats induced cryptorchidism, hypospadias, abnormal Leydig cell aggregation, multinucleated gonocytes, and decreased testicular testosterone level at fetal stage^{1–8)} and induced infertility at adult^{9, 10)}. Testicular atrophy, reduction of testicular mRNA expression of steroidogenesis related genes, and infertility have been induced by oral administration of di(2-ethylhexyl) phthalate (DEHP) in prepubertal male rats^{11–13)}, and testicular damage caused by DEHP showed age-specific in rats¹⁴⁾. Furthermore, mono(2-ethylhexyl)phthalate

(MEHP), a monoester metabolic product of DEHP, also induced the disorder of testicular structures due to cell death and sloughing of spermatogenic cells in prepubertal laboratory animals^{11, 15–19)}. Phthalate esters including DiBP are rapidly hydrolyzed to monoester by hepatic and intestinal non-specific hydrolases in a variety of animal species²⁰⁾. It has been speculated that the different effects of each phthalate ester among each animal species have a relationship with the structure of the butyl moiety in the side chain of phthalate monoesters²¹⁾. Di-iso-butyl phthalate (DiBP) [C₁₆H₂₂O₄-C₆H₄CO₂CH₂CH(CH₃)₂]₂ has similar application properties to DBP and is substituted for DBP. Thus, DiBP has been used in polyvinyl chloride (PVC), inks, paints, adhesives and cosmetics²²⁾. Although DiBP has been frequently used, for example, 100 tons per year in Denmark²³⁾, only a few reports on the effects of DiBP on male reproductive system are available at pres-

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ent. Oishi and Hiraga (1980a) demonstrated that DiBP reduced body weight and Zinc concentration in young mouse testes by feeding a diet containing 2% DiBP. Furthermore, skeletal malformations, undescended testes, reduced anogenital distance and testicular histopathological effects were observed by administration of DiBP in rats at fetal stage^{23, 24}. Therefore, in order to clarify the effects of DiBP on testes in detail, the present study was carried out using prepubertal rats and mice, especially focusing on apoptotic spermatogenic cells.

Material and Methods

Test compounds: DiBP, di-iso-butyl phthalate standard, CAS No.84-69-5, purity 99.9%, and corn oil were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and concentrations of DiBP: Twenty-one-day-old Sprague-Dawley (SD) male rats and C57Bl/6N male mice were purchased from Charles River, Co., Japan. For an acute exposure experiment, they were once given DiBP at various concentrations (100, 300, 500, 800, or 1,000 mg/kg) in corn oil by oral gavage, and 1 day later, the animals were sacrificed under anesthesia by diethyl ether. For a subchronic exposure experiment, they were daily given DiBP at various concentrations (100, 300, 500, 800, or 1,000 mg/kg) for consecutive 7 days in corn oil by oral gavage, and 1 day later (after the last administration), they were sacrificed as well. For a recovery experiment, SD male rats were once given DiBP (1,000 mg/kg), and were sacrificed at 1 day (D1) to D8 after administration. The concentrations of each experiment were based on the findings of several previous studies on MEHP and DBP^{11, 17, 25}. Animals were given DiBP in corn oil at a volume equal to 4 ml/kg. Control groups were received the same volume of corn oil. Then, the testes in each experiment were excised and fixed in 4% paraformaldehyde (PFA) in 0.01 M phosphate buffered saline (PBS) at 4°C. Thereafter, they were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Paraffin blocks were cut at 5 µm in thickness.

All procedures were carried out in accordance with a protocol approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

In situ TUNEL method: Apoptosis of spermatogenic cells was examined using the TUNEL method according to the protocol of the 'in situ apoptosis detection kit' (TaKaRa, Tokyo, Japan). The sections were deparaffinized, rehydrated, and then predigested with 10 µg/ml protease K for 15 min. Thereafter, they were incubated in PBS containing 3% H₂O₂ for 15–30 min to block endogenous peroxidase activity. Then, they were incubated with a fluorescein isothiocyanate (FITC)-labeled TdT

enzyme in a humidified chamber at 37°C for 90 min. After washing, the sections were incubated with anti-FITC horseradish peroxidase (HRP) conjugate at 37°C for 30 min. Finally, TUNEL-positive cells were detected by 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Wako, Tokyo, Japan) substrate, counterstained with methyl green dye, mounted, and observed using an optical microscope for counting the apoptotic cell number per each seminiferous tubule.

Immunohistochemistry: Rats were daily given DiBP (500 mg/kg) for consecutive 7 days, and sacrificed. Thereafter, testes were excised, fixed, and embedded in paraffin, as above mentioned. For vimentin immunohistochemistry, paraffin sections were deparaffinized, rehydrated, and later heated in 0.01 M citrate buffer (pH 6.0) for 10 min in a microwave to facilitate antigen retrieval. Then, they were treated with 3% peroxidase for 30 min to eliminate endogenous peroxidase and blocked with a TNB blocking buffer. A monoclonal anti-vimentin antibody (mouse immunoglobulin M [IgM] isotype, clone LN-6; Sigma, St. Louis, MO, USA) at a dilution 1:100 was used for staining vimentin in seminiferous tubules. The sections were fixed in 4% PFA for 24 hr, rinsed in PBS, blocked with a TNB (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% Blocking Reagent, pH 7.5) blocking buffer, supplied in TSA (Tyramide Signal Amplification) Biotin System kit (Perkin Elmer Life Sciences, Boston, MA, USA), and incubated with primary antibody at 4°C overnight. Thereafter, they were incubated with biotinylated goat anti-mouse IgG secondary antibody for 1 hr at room temperature, followed by the ABC kit (Funakoshi, Tokyo, Japan). The immunoreactions were visualized with 0.05% DAB (Wako) with H₂O₂ in PBS. The sections were counterstained with methyl green dye, and observed by light microscopy.

Statistical analysis: Only intact and round seminiferous tubules were selected for counting. The number of TUNEL-positive cells was counted in 15–20 randomly-selected round seminiferous tubules from each animal of all control and treated groups. The data, calculated as a percentage of total, are expressed as mean±S.E.M. Statistical analysis was conducted using the Student's paired *t*-test. The *p*-values less than 0.05 were considered statistically significant. Asterisks indicate a statistically significant difference compared to the control, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Results

Acute exposure experiment: In the acute experiment, the administration of DiBP did not significantly reduce the testis weight at any concentrations in rats (Fig. 1a). Similarly, no significant reduction of the testis weight also occurred in mice (Fig. 1c). In rats, the number of TUNEL-positive spermatogenic cells showed a signifi-

Fig. 1. Acute exposure experiment. Changes in testis weight (a, c) and TUNEL-positive cells in testes of rats (A–D, b) and mice (E–H, d) after DiBP administration. Animals exposed to 0 (A, E), 500 (B, F), 800 (C, G), or 1,000 mg/kg (D, H) of DiBP. Testis weight did not reduce in both mice and rats (a, c) after DiBP administration. Ratio of TUNEL-positive cells increased ($p < 0.001$) in the DiBP treated rats (B, C, D, b) at higher (≥ 500 mg/kg) concentrations. Mice showed a tendency to increase ($p = 0.071$) apoptotic spermatogenic cells (G, d) only in the 800 mg/kg DiBP treated group. Data represent means $\pm$ S.E. bar = 100 μ m.

cant increase at 24 hr in the 500, 800, and 1,000 mg/kg ($p < 0.001$) DiBP treated groups, compared to the control (Fig. 1B, C, D, b). As shown in Fig. 1b, it increased in a dose-dependent manner. However, in mice, TUNEL-positive cells increased only in the 800 mg/kg DiBP ($p = 0.071$) treated group (Fig. 1G, d).

Subchronic exposure experiment: In the subchronic experiment, the animals were daily given DiBP of each concentration for consecutive 7 days. In rats, a significant loss of the testis weight was found ($p < 0.001$) in the 500, 800, and 1,000 mg/kg/day treated groups, compared to the control (Fig. 2a), whereas in mice, only

the 1,000 mg/kg/day treated group revealed a significant reduction ($p < 0.01$) (Fig. 2c). The number of TUNEL-positive spermatogenic cells in rats were significantly ($p < 0.05$) increased in the 500, 800, and 1,000 mg/kg/day DiBP treated groups, compared to the control (Fig. 2B, C, D, b), but not in the 100 and 300 mg/kg/day treated groups. In mice, no significant increase in the number of TUNEL-positive spermatogenic cells was found at any concentrations (Fig. 2d).

Recovery experiment after a single administration of DiBP: The recovery experiment was carried out in rats. Although the testis weight showed no significant loss at

Fig. 2. Subchronic exposure experiment. Changes in testis weight (a, c) and TUNEL-positive cells in testes of rats (A–D, b) and mice (E–H, d) after DiBP administration for consecutive 7 days. Animals exposed to 0 (A, E), 500 (B, F), 800 (C, G), or 1,000 mg/kg/day (D, H) of DiBP. Testis weight reduced in rats in the 500, 800, and 1,000 mg/kg/day DiBP treated groups (a). Only the 1,000 mg/kg DiBP treated group showed a significant testis weight loss ($p < 0.01$) compared to the control (c). TUNEL-positive cells increased in parallel with testis weight loss in rats (b). No significant changes in the number of TUNEL-positive cells were observed in mice (d). Data represent means $\pm$ S.E. bar = 100 μ m.

1 day (D1) after administration of 1,000 mg/kg DiBP, it reduced significantly at D2 ($p < 0.05$) and D5 ($p < 0.001$), compared to the control. Thereafter, it recovered to normal level at D6 and D8 (Fig. 3a). TUNEL-positive spermatogenic cells still revealed an increase in number at D1, D2, and D5. While, they abruptly decreased, and became almost the same with those in the control at D6 and D8.

Distribution of vimentin filaments: Vimentin filaments were expressed both in the control (Fig. 4A) and 500 mg/kg/day DiBP treated rats for 7 consecutive days

(Fig. 4B). In the control, vimentin appeared obviously in the perinuclear and basal regions of Sertoli cells, and it extended towards the lumen (Fig. 4A). No TUNEL-positive spermatogenic cells were detected in the seminiferous tubule of the control (Fig. 4C). In the DiBP treated rat, vimentin filaments were partly disorganized or disappeared in the perinuclear and basal regions of Sertoli cells. Sloughing of TUNEL-positive spermatocytes from the epithelium was observed in the treated group as shown in Fig. 4D.

Fig. 3. Recovery experiment. Light micrographs and diagrams showing the changes in TUNEL-positive cells at D1 (A), D5 (B), D6 (C) and D8 (D) after DiBP administration in rats. Inset shows an enlarged view of each section. Comparison of the testis weight of the control and 1,000 mg/kg DiBP treated rats from D1 to D8 (a,b). Testis weight in the treated groups was lower than that in the control group (a). A recovery ($p < 0.001$) occurred at D6 (b). Data represent means $\pm$ S.E. bar = 100 μ m.

Discussion

The present study illustrates that DiBP induces apoptosis in spermatogenic cells in 21-day-old rats via acute or subchronic oral administration. Since oral administration of DiBP at higher concentrations can elicit a damage in prepubertal rat testes, it is obvious that DiBP reveals a testicular toxicity, similar to other phthalate esters as previously reported^{10, 23, 24, 26, 27}. In contrast, a significant increase of apoptotic spermatogenic cells could not be observed in prepubertal mouse testis, even at higher concentrations and consecutive exposure for 7 days. Five-week-old rats²⁸ and mice²⁹ exposed to 2% DiBP in diet showed a loss of testis weight only in rats. The patterns of responses to testicular oxidative stress seem to be species-specific³⁰. Although in some previous studies, the different specificity between mice and rats was confirmed by administration of the same chemical agent^{31–33}, the detailed mechanism of this species-difference is still thought to be equivocal. Kluwe *et al.* (1982) elucidated that DEHP caused liver tumors in both mice and rats, but di (2-ethylhexyl) adipate (DEHA) triggered liver tumors only in mice, not in rats. In addition,

Murakami *et al.* (1995) showed that after administration of procymidone for 3 months, LH levels in serum, testis and pituitary were increased for 13 weeks in rats, while for 4 weeks in mice. In contrast to rats, no significant increase in testosterone occurred in mice either *in vivo* or *ex vivo* during the course of the study. They concluded that the difference between mice and rats appeared in response of Leydig cells to LH stimulation in procymidone administration. Furthermore, the difference in response to trichloroethylene (TCE, a well-known carcinogen in laboratory animals) between mice and rats is suggested to be due to the difference in metabolism³⁴. But, the precise mechanism involved in is not yet clearly defined³⁵. Our previous studies showed that MEHP, a metabolic product of DEHP, significantly induced spermatogenic cell apoptosis in both mouse and rat testes^{5, 17–19}. Conversely, in this study, DiBP affected testes only in rats. Although the chemical structure of DiBP is similar to that of DEHP, the present study assumes that the effects of DiBP on testes are species-specific. Furthermore, the testicular response to oral administration of DEHP may be due to pharmacokinetic rather than tissue sensitivity difference¹⁴, and even in the same species, an age-specific^{14, 36}

Fig. 4. Distribution of vimentin filaments in Sertoli cells and TUNEL-positive cells in the control (A, C) and the DiBP treated group (B, D). Vimentin filaments were partly disorganized or disappeared in Sertoli cells. bar = 100 μ m.

or sexual-specific³¹⁾ effect is also confirmed. Moreover, the initiation of spermatogenic cell proliferation at fetal stage is different between mice and rats³⁷⁾, and the length of spermatogenic cell cycle at adult is 12.9 days in rats³⁸⁾ and 8.6 days in mice³⁹⁾. These differences may contribute to the different results between mice and rats in the present study to some extent.

The population of TUNEL-positive spermatogenic cells reduced in a time-dependent manner in rats in the recovery experiment after a single administration of 1,000 mg/kg DiBP. The abrupt increase of TUNEL-positive spermatogenic cells was observed at D1–D5, and then the significant regression occurred at D6. In the MEHP recovery experiment, TUNEL-positive cells also reduced in a time-dependent manner in 21-day-old mice, but the rate of testis weight gain was lower compared to the control from D1 to D9¹⁸⁾. After administration, 80%–90% of MEHP is excluded from the body within 24 hr^{11, 40)}, indicating that even though MEHP is almost eliminated from the body, its effects can still persist in testes¹⁸⁾. By MEHP administration, the number of TUNEL-positive cells gradually decreased from D3 to D7, and reached the same amount with the control at D9. While, DiBP caused a significant loss of testis weight at D2 and D5, and then the testis weight returned to the normal level at D6, compared to the control. Similarly, the number of TUNEL-positive cells decreased and returned to the baseline as the control at D6.

To our knowledge, no data are available on the elimination time of DiBP from the body. From our previous and present data, it seems that the testicular recovery in DiBP exposure is earlier than that in MEHP exposure. In other words, the testicular toxic effects of DiBP are

weaker than those of MEHP. This supports the view that MEHP triggers a sequence of disruptive events in testes, which still persists after elimination of MEHP¹¹⁾, but persists not so long in DiBP.

Although the apoptotic spermatogenic cells induced by phthalate esters were detected with in situ TUNEL method. TUNEL-positive labeling was not found in Sertoli cells in rats^{41–43)} and mice^{17–19, 44)}. In addition, both in vivo and in vitro experiments demonstrated that the Sertoli cell is the primary site of phthalate-induced testicular toxicity⁴⁵⁾. Sertoli cell alterations, including vacuolization, inhibition of transferring secretion and etc., can induce spermatogenic cell apoptosis⁴¹⁾. This prompted us to focus our attention on primary effects of DiBP on Sertoli cells. Previous experiments demonstrated that MEHP causes the alternation of Sertoli cell cytoskeleton in mice and rats, particularly in distribution of vimentin filaments^{19, 41)}. This study showed that vimentin filaments were partly disorganized or disappeared in the perinuclear and basal regions of Sertoli cells in the DiBP treated rat. At the same time, sloughing of TUNEL-positive spermatogenic cells from the seminiferous epithelium was observed. Since vimentin filaments of Sertoli cells play an important role in the maintenance of spermatogenesis⁴⁶⁾, our finding suggests that consecutive exposure of DiBP can induce the conspicuous alterations in distribution of vimentin filaments, and it correlates with sloughing of spermatogenic cells from the seminiferous epithelium. From the present recovery experiment, vimentin filaments can be reorganized after elimination of DiBP, and then spermatogenic cells repopulate and are restored⁴⁶⁾.

In conclusion, our present study showed that DiBP caused a significant increase of TUNEL-positive spermatogenic cells and a disorder of vimentin filaments in rats. These toxic effects are reversible, and testes recover to normal until day 6 after administration. We suggest that DiBP can be a lower-potent toxic agent than MEHP and reveals a species-specific toxicity.

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