論文の内容の要旨

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論文題目: Toxic effects of di-iso-butyl phthalate on testes in prepubertal rats and mice *in vivo* and *in vitro*

（*In vivo* および *In vitro* 実験系における Di-iso-butyl phthalate の未成熟ラットおよびマウス精巣への毒性作用）

DiBP, one of phthalate esters, has been used as a plasticizer to produce many common consumer products. The metabolites of such phthalate esters are released into the environment, and pose potential health risks for humans and wildlife. Since the effects of DiBP on testes still remained to be obscure, the present study focused on the testicular toxicity of DiBP, and was conducted to clarify its precise mechanism.

In Chapter Ⅰ, the effects of DiBP by acute and subchronic administration on testes in prepubertal rats and mice were examined, and a recovery experiment was also carried out. Twenty-one-day-old Sprague-Dawley (SD) male rats and C57Bl/6N male mice were used for these experiments. For an acute experiment, they were once given DiBP at various concentrations (100, 300, 500, 800, or 1000 mg/kg) by oral gavage and were sacrificed under anesthesia by diethyl ether 1 day later. For a subchronic experiment, they were daily given DiBP at various concentrations (100, 300, 500, 800, or 1000mg/kg) for consecutive 7 days, and 1 day later (after the last administration), they were sacrificed as well. The results showed that DiBP increased TUNEL-positive (apoptotic) spermatogenic cells in prepubertal rats testes via acute or subchronic oral administration. Especially, the administration of DiBP at higher concentrations (≥500mg/kg) elicited a severe damage in

prepubertal rats testes. Thus, it is obvious that DiBP reveals a testicular toxicity, similar to other phthalate esters as previously reported. In contrast, a significant increase of apoptotic spermatogenic cells could not be observed in prepubertal mice testes, even at higher concentrations and consecutive exposure for 7 days. This discrepancy suggests that DiBP has a species-specific toxicity. For a recovery experiment, SD male rats were once given DiBP (1000mg/kg), and were sacrificed at 1 day (D1) to D8 after administration. 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. In case of di(n-ethylhexyl)phthalate (DEHP), after administration, 80%~90% of DEHP is excluded from the body within 24 hr, indicating that even though DEHP is almost eliminated from the body, its effects can still persist in testes. By DEHP administration, the number of TUNEL-positive cells gradually decreased from D3 to D7, and reached the same with that in 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. It seems that the testicular recovery in DiBP exposure is earlier than that in DEHP exposure.

In Chapter Ⅱ, to clarify the mechanism of DiBP effects on prepubertal rats testes, some steroidogenic enzymes mRNA expressions, and intratesticular testosterone (ITT) concentrations in rats testes exposed to DiBP or estradiol-3-benzoate (EB) were examined. Previous studies showed that hormone controls spermatogenesis, and elevation of testicular apoptosis is due to androgen suppression and exposure to xenoestrogen. While, other studies hypothesized that induced spermatogenic cell apoptosis by exposure to phthalate esters is not caused by change in ITT level associated with steroidogenesis-related genes expression *in vitro*. First, to clarify whether testicular histopathological (increase of TUNEL-positive spermatogenic cells) effects elicited by DiBP in rats is attributed to mimic effects of estrogenic function, the animals were exposed to pure estrogen receptor (ER) antagonist, ICI 182,780, prior to DiBP or EB administration. The results showed that the number of apoptotic cells was not rescued by inhibiting the reaction of ER. This finding indicates that the phenomenon of increased spermatogenic cell apoptosis by exposure to DiBP might not occur via the same interaction route or molecular signal pathway with estrogen. Moreover, apoptotic spermatogenic cells showed high rate in both DiBP and EB subchronic (7 days) exposure groups, whereas, after acute (6 hr) exposure to EB, the incidence of apoptotic cells seem to be less than that in the DiBP treated group. This evidence also indicates that DiBP might elicit their adverse effects through different signal pathway from EB. Additionally, although the ITT concentration was decreased at 6 hr and 24 hr after EB administration, it was restored in the DiBP treated group at 24 hr after administration, and interestingly, the incidence of apoptosis in testes revealed a slight increase at 24 hr in the DiBP treated group. These results indicate that 1) increased number of apoptotic spermatogenic cells was not directly related with ITT concentration; and 2) EB may affect

hormonal systemic circulation, but DiBP seems not to disturb the endocrine circulation. Since the ITT concentration was repressed in the DiBP and EB treated groups, steroidogenic enzymes (*P450scc*, *P450c17*, *3β-HSD* and *17β-HSD)* mRNA expressions were examined by using reverse transcription-polymerase chain reaction (RT-PCR). *P450scc P450c17* and *17β-HSD* genes expressions were decreased in both DiBP and EB treated groups, while *3β-HSD* was decreased only at 6 hr in the DiBP treated groups but not in the EB treated group. Therefore, it is suggested that the effect of DiBP on testicular steroidogenesis is different from that of EB. Additionally, a number of general factors (*FasL, caspase-3, vimentin*) that involved in apoptotic signal pathway were examined in order to compare the effects of DiBP with anti-androgen (flutamide) or xenoestrogen (EB) by immunohistochemistry. The results showed that the expression in all apoptotic factors in the treated testicular tissues was increased compared to that in the control. It seems that testicular apoptosis induced by DiBP is through well-known apoptotic signal pathway.

In Chapter Ⅲ, *in vitro* studies using testicular tissues and Sertoli cell cultures were carried out to clarify whether DiBP affects them directly. Several studies evaluated that phthalate esters could exert their testicular toxic effects directly on cultured cells. Moreover, it is reported that estrogen induces spermatogenic cell apoptosis *in vitro*. These findings indicate that increased apoptosis of spermatogenic cells may not be attributed to disruption of the hypothalamo-pituitary-testicular axis. In *in vitro* studies, DiBP and 17β-estradiol (E_2) were dissolved in pure ethanol (99.9%), and then diluted to various required concentrations by nutrient medium. Both samples were incubated at 32℃, 5%CO_2, and harvested at 3 and 12 hr after initial administration. The rate of spermatogenic cell apoptosis was determined by TUNEL analysis, and steroidogenic enzymes mRNA expressions were also examined by RT-PCR. Apoptotic spermatogenic cells were frequently encountered at 12 hr after administration compared to those in the control. The mRNA level of testicular steroidogenic genes revealed no change in all analyzed samples. Immunohistochemistry showed the disruption of vimentin filaments at high concentrations of DiBP and E_2. These *in vitro* experiments demonstrated that DiBP and also estrogen induce spermatogenic cell apoptosis, suggesting that DiBP acts directly on testes.

In conclusion, DiBP shows a species-specific toxicity. It affects testes directly and induces spermatogenic cell apoptosis with no association with ITT concentration. This study suggests that the adverse effects of DiBP differ in several points from estrogen.