K-ras mutation

Mutations in the *ras* oncogene have been identified in approximately 20% of endometrioid carcinomas but not in serous tumors in women [39-43]. In rodents, *K-ras* point mutations have also been detected in endometrial hyperplasia and adenocarcinomas but not in normal endometrium in Donryu rats [43], suggesting that alteration in the *K-ras* gene may be an important initiating event in this strain of rats, comparable to the endometrioid carcinoma in the human case.

P53 mutation

Mutations in the p53 tumor suppressor gene and accumulation of p53 protein have been detected in approximately 90% of serous carcinomas [45, 46], whereas they are comparatively rare in endometrioid carcinomas and AH [47]. In Donryu rats, such changes are only features of poorly-differentiated adenocarcinomas [11], although mutations have been detected in some well-differentiated endometrial adenocarcinomas in F344 rats [48]. These results suggest that p53 mutation might be late stage event of uterine carcinogenesis or dedifferentiation in Donryu rats, but not in F344 rats, suggesting the profile in former rat strain to be similar to the endometrioid type and the latter to the serous one.

PTEN (phophatase and tensin homolog) mutation

PTEN is a tumor suppressor gene and its mutation as well as loss of PTEN protein are suggested to be early events in uterine carcinogenesis in women [49]. However, in Donryu rats, immunohistochemical results and/or mRNA expression profiling of uterine proliferative lesions which were laser-microdissected from ethanol-fixed, paraffin-embedded tissues did not show any decrease or loss of PTEN [11], suggesting no relation with endometrial adenocarcinoma development in this rat.

β-Catenin mutation

Nuclear accumulation of β-catenin in atypical hyperplasias and carcinomas is more frequent than in simple/complex hyperplasias of the endometrium in women [49] but in rats no nuclear accumulation of β-catenin could be detected in any uterine proliferative lesions immunohistochemically [11].

To conclude, findings for expression and mutation of various genes during endometrial adenocarcinoma development are summarized in Figure 8. Unfortunately, useful early endpoint markers based on molecular biology have yet to be established for uterine cancer, and this needs to be a focus of future research.

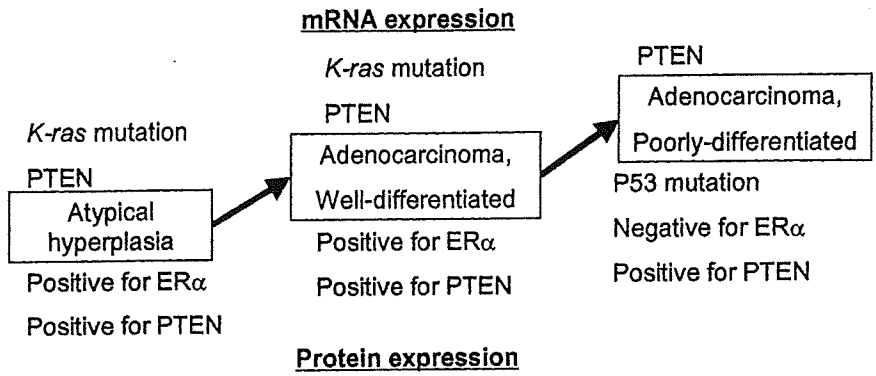

Figure 8. Gene and protein expression profiles for different stages of uterine carcinogenesis in the Donryu rat. ERα, estrogen receptor α.

References

1. Sherman, M.E. 2000, Modern Pathol., 13, 295.
2. Katsuda, S., Yoshida, M., Kuroda, H., Ando, J., Takahashi, M., Kurokawa, Y., Watanabe, G., Taya, K., and Maekawa, A. 2002, Jpn. J. Cancer Res., 93, 117.
3. Maekawa, A., Takahashi, M., Ando, J., and Yoshida, M. 1999, J. Toxicol. Pathol., 12, 1.
4. Nagaoka, T., Takeuchi, M., Onodera, H., Mitsumori, K., Lu, J., and Maekawa, A. 1993, In vivo, 7, 525.
5. Nagaoka, T., Onodera, H., Matsushima, Y. Todate, A., Shibutani M., Ogasawara, H., and Meakawa, A. 1990, J. Cancer Res. Clin. Oncol., 116, 623.
6. Niwa, K., Murase, T., Mirishita, S., Tanaka, T., Mori, H., and Tamaya, T. 1993, Jpn. J. Cancer Res., 84, 951.
7. Niwa, K., Tanaka, T., Yokoyama, Y. Mori, H., and Tamaya, T. 1991, Jpn. J. Cancer Res., 82, 1391.
8. Maekawa, A., Onodera, H., Tanigawa, H., Furuta, K., Kanno, J., Matsuoka, C., Ogiu, T., and Hahashi, Y. 1986, Jpn J Cancer Res. (Gann), 77, 882.
9. Nagaoka, T., Takeuchi, M., Onodera, H., Matsushima, Y., Ando-Lu, J., and Maekawa, A. 1994, Toxicol. Pathol., 22, 261.
10. Pecorelli, S., Benedet, J.L., Creasman, W.T., and Shepherd, J.H. 1999, Int. J. Gynecol. Obst., 64, 5.
11. Yoshida, M., Shimomoto, T., Hatanaka, Y., Mihara,T., Maekawa, A., and Nakae, D. 2004, 181 Joint international meeting of the Japanese Society of Toxicologic Pathology & the International Federation of Societies of Toxicologic Pathology; Co-sponsored by the International Academy of Toxicologic Pathology (Program and abstracts).
12. Pertschuk, L.P., Masood, S., Simone, J., Feldman, J.G., Fruchter, R.G., Axiotis, C.A., and Greene, G.L. 1996, Gynecol. Oncol., 63, 28.
13. Susumu, N., Aoki, D., Suzuki, N., and Nozawa, S. 2001, Jpn. J. Cancer Chemother., 28, 934-45 (Abstract in English),
14. Ando-Lu, J., Takahashi, M., Imai, S., Ishihara, R., Kitamura, T., Iijima, T., Takano, S., Nishiyama, K., Suzuki, K., and Maekawa, A. 1994, Jpn. J. Cancer Res., 85, 789.

15. Katsuda, S., Yoshida, M., Saarinen, N., Smeds, A., Nakae, D., Santti, R., and Maekawa, A. 2004, Exp. Biol. Med., 229, 417.
16. Nishiyama, K., Ando-Lu, J., Nishimura, S., Takahashi, M., Yoshida, M., Sasahara, K., Miyajima, K., and Maekawa, A.1998, In Vivo, 2, 363.
17. Yoshida, M., Kudoh, K., Katsuda, S., Takahashi, M., Ando, J., and Maekawa, A. 1998, Cancer Lett., 134, 43.
18. Abbott, D.H., Dumesic, D.A., and Franks, S. 2002, J. Endocriol., 174, 1.
19. Katsuda, S., Yoshida, M., Isagawa, S., Asagawa, Y., Kuroda, H., Watanabe, T., Ando, J., Takahashi, M., and Maekawa, A. 2000, Reprod. Toxicol., 14, 119.
20. Yoshida, M., Katsuda, S., Ando, J., Kuroda, H., Takahashi, M., and Maekawa, A. 2000, Toxicol. Lett.,116, 89.
21. Yager, J.D., and Liehr, J.G. 1996, Ann. Rev. Pharmacol. Toxicol., 36, 6535.
22. Liehr, J.G., Ricci, M.J., Jefcoate, C.R., Hannigan, E.V., Hokanson, J.A., and Zhu, B.T. 1995, Proc. Natl. Acad. Sci. USA, 92, 9220.
23. Liehr, J.G., and Ricci, M.J. 1996, Proc. Natl. Acad. Sci. USA, 93, 3294.
24. Hammond, D.K., Zhu, B.T., Wang, M.Y., Ricci, M.J. and Liehr, J.G. 1997, Toxicol. Appl. Pharmacol., 145, 54.
25. Badawi, A.F., Cavalieri, E.L., and Rogan, E.G. 2000, Carcinogenesis, 21, 1593.
26. Liehr, J.G. 2000, Endocrine Reviews, 21, 40.
27. Liehr, J.G., Fang, W.F., Sirbasku, D.A., and Ulubelen. 1986, J. Steroid Biochem., 24, 353.
28. Newbold, R.R., and Lier, J.G. 2000, Cancer Res., 60, 235.
29. Yoshida, M., Katashima, S., Ando, J., Tanaka, T., Uematsu, F., Nakae, D., and Maekawa, A. 2004, Carcinogenesis, 11, 2257.
30. Horn, T.L., Reichert, M.A., Bliss, R.L., and Malejka-Giganti, D. 2002, Biochem. Pharmacol., 64, 393.
31. Jang, E.H., Park, Y.C., and Chung, W.G., 2004, Food Chem. Toxicol., 42, 1749.
32. Jongen, W. 1996, Proc. Nutr. Soc., 55, 433.
33. Bonnesen, C., Eggleston, I.M., and Hayes, JD. 2001, Cancer Res., 61, 6120.
34. Bradlow, H.L., Michnovicz, J.J., Telang, N.T., and Osborne, M.P. 1991, Carcinogenesis, 12, 1571.
35. Kojima, T., Takana, T., and Mori, H. 1994, Cancer Res., 54, 1446.
36. Meng, Q., Yuan, F., Goldberg, I.D., Rosen, E.M., Auborn, K., and Fan, S. 2000, J. Nutr., 130, 2927.
37. Michnovicz, J.J., and Bradlow, H.L. 1990, J. Natl. Cancer Inst., 82, 947.
38. Michnovicz, J.J., Adlecreutz, H., and Bradlow, H.L. 1997, J. Natl. Cancer Inst., 89, 718.
39. Boyd, J., and Risinger, J.I. 1991, Mol. Carcinogenesis, 4, 189.
40. Caduff, R.F., Johnson, C.M., and Frank, T.S. 1995, Am. J. Pathol., 146, 182.
41. Enomoto, T, Inoue, M, Perantoni, A.O., Buzard, G.S., Miki, H., and Tanizawa, O., and Rice, J.M. 1991, Cancer Res., 51, 5308.
42. Ignar-Trowbridge, D., Risinger, J.I., Dent, G.A., Kohler, M, Berchuck, A., McLachlan, J.A., and Boyd, J. 1992, Am. J. Obstet. Gynecol., 167, 227.
43. Sasaki, H., Nishii, H., Takahashi, H., Tada, A, Furusato, M., Terashima, Y., Siegal, G.P., Parker, S.L., Kohler, M.F., Berchuck, A., et al. 1993, Cancer Res., 53, 1906.
44. Taniguchi, K., Yaegashi, N., Jiko, K., Maekawa, A., Sato, S., and Yajima, A. 1999, Tohoku J. Exp. Med., 189, 87.

45. Demopoulos, R.I., Mesia, A.F., Mittal, K., and Vamvakas, E. 1999, Int. Gynecol. Pathol., 18, 233.
46. Tashiro, H., Isacson, C., Levine, R., Kurman, R.J., Cho, K.R., and Kedrick, L. 1997, Am. J. Pathol., 150, 177.
47. Sherman, M.E., Bur, M.E., and Kurman, R.J. 1995, Hum. Pathol., 26, 1268.
48. Nagaoka, T., Miyajima, H., and Maekawa, A. 2004, 180, Joint international meeting of the Japanese Society of Toxicologic Pathology & the International Federation of Societies of Toxicologic Pathology; Co-sponsored by the International Academy of Toxicologic Pathology (Program and abstracts).
49. Saegusa, M., Hashimura, N, Yoshida, T., and Okayasu, I., 2001, Br. J. Cancer, 84, 209.

雑　誌

SPRINGER REVIEWS

井上 達／井口泰泉 編

生体統御システムと内分泌撹乱

Springer
シュプリンガー・フェアラーク東京

はじめに——発刊にあたって——

環境化学物質が生体のホルモン受容体との相互作用によりホルモン様の作用をひき起こしたり，あるいは抑制することが指摘されて久しい．それらの可能性の一般化に対して真っ向から疑問をもつ人々がいた一方で，ありそうな現象ながら，実環境でそれを検証することのむずかしさも想定され，当初，基礎科学が入り込む余地は甚だ乏しいようにも思われた．

この間，そうした外来異物の受容体を介した生体影響については，従前対象にならなかった低用量作用の有無をはじめ未知の事柄も少なくなかった中で，内在性ホルモン・植物ホルモン・その他の外来異物相互の競合的相互作用，アリール炭化水素受容体を含む核内受容体シグナルのプレイオトロピックな相互作用，などと，次々問題の要素が明らかになってきた．併せて近年の膜受容体の発見は，この課題の認識をノン・ジェノミックな現象にまで拡げた．こうした中で，環境化学物質のヒトへの影響が日常曝露の限りでは蓋然性にとどまることが明らかになった一方，低用量の胎児期曝露による次世代影響研究の基礎生物学的な実験的意義はむしろ高まってきた．

日本は，かつて国内の女性がジエチルスチルベストロール(DES)による発がんの惨禍を免れる原点となった研究で知られる学者Takasugiを輩出しており，また，最近の研究をみても，わが国の数々の研究者の活躍には他にひけをとらないめざましいものがある．若手研究者の本邦におけるこの領域の研究において，さらなる進展の期待される所以である．ときあたかも世界保健機関は，内分泌撹乱現象と該当物質について地球規模での既存報告の分析を行った．そこで強調された点は，生物学的蓋然性とよぶ概念からなる生物全体を俯瞰した基礎生物学的にして本質的な危惧であった．以前なら研究の動機づけとしても，とても十分とは思われない生物学的蓋然性が，グローバル・アセスメントの内分泌撹乱現象の確認と今後の積極的な研究の必要性への勧告に結びつき得た背景には，序論で井口によって紹介されているように，広汎な生物界全体での検証作業が進んだことと相まって，種々の生物で全ゲノム配列の解読を終了させた基礎生物学の進展が基盤になっていたと考えられる．

このような流れの中で，サイエンスとしていま最も注目されている点が，生体異物の胎児・新生児への影響であり，しかもその高次生命系，すなわち神経系，免疫系，および内分泌系といった，生体統御システムとしてのネットワーク機能への影響であり，これに起因する次世代への影響についての研究である．しかしながら，初期胚の形態形成にかかわる分子発生学領域の研究はたしかにめざましい進展の佳境にあるとはいえ，これに引き続く高次生命系の形態機能

形成過程には未知の事柄が山積しているので，この研究はたやすいものであるとは思われない．課題のおかれている位置づけとしても，過去に看過されてきた長期にわたる影響を対象とした新しい研究領域である．これはむしろ本書の読者として想定されている若い研究者に託された，新世紀の皮切りにふさわしい難題といって過言ではない．歴史的に未知の生体機能は，少なからず"異常反応"との対比の中で解明されてきた．このような背景の中で，内分泌器官と相互作用する化学物質の高次系への影響は，かぎりない未知の生体機能の解明の可能性を秘めた，科学的興味の焦点となりつつあるのである．

このたび，以上のような事情から井口教授との共編の形で，『生体統御システムと内分泌撹乱』の出版が実現することとなった．この出版の目的は，内分泌器官と相互作用をもち，内分泌作用の調節機構を"撹乱"する可能性をもついわゆる内分泌撹乱化学物質の生体影響について，極力，基礎科学的に生体統御システム生物学に焦点を絞った実験的研究の解説書をまとめ，大学院生，若手研究者のこの領域の問題に対する学問的関心に応えることにある．いわゆる内分泌撹乱問題が，少なからず興味本位に，かつ恣意的に取り上げられてきた中で，その生体統御システムとしての高次生命系での振る舞いに注目し，未知で興味深い研究対象として，この領域の基礎研究者が正面からこれに取り組んだ解説書はいまだにみられない．本書では，これを埋め合わせ，この領域が内包する多くの課題をそれぞれの専門分野から明らかにしようと試みた．

本編が若手研究者の将来に向けての研究にいささかなりとも示唆的なものを提供することとなれば，編者にとっては望外の喜びであり，また執筆にご快諾下さった先生方におかれても，思いを等しくするところと信ずる．そして，新しい研究者たちがこの領域の研究へと，怒濤のような勢いであらたに参画する弾みとなるならば，至福の喜びである．

2005年4月

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なお，本書の執筆にあたっては，編者側からは必ずしも内分泌撹乱化学物質の定義を特定はしなかったが，世界保健機関は，これを次のように提案している．

"内分泌撹乱物質とは，健常な生物またはその子孫，あるいは個体群における内分泌系の機能を変質させ，有害な影響を生じさせる外因性物質，または混合物である．"

また，本書の作製にあたり，以下の5人の先生方にご校閲をいただいた．記してお礼申し上げる．

黒田洋一郎(東京都神経科学総合研究所)，紫芝良昌(前・虎の門病院)，長尾哲二(近畿大学)，長濱嘉孝(岡崎統合バイオサイエンスセンター)，吉田　緑(佐々木研究所)　　(敬称略)

(本書の各章の著述の基礎となった諸研究，ならびに当出版の一部は，厚生労働省科学研究費の研究補助金[H13 - 生活 - 013]に基づいている．)

Environ. Mutagen Res., 27: 133-137 (2005)

OECDガイドラインにおける動物福祉

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Animal welfare mind and achievement in the OECD Guidelines for Testing of Chemicals

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Summary

The OECD established the Guidelines for Testing of Chemicals in 1981, which is the basis of the mutual acceptance of data (MAD) system among the member countries to prevent unnecessary repetition of toxicity tests, and consequently to reduce the number of animals used. The Guidelines was soon subjected to revision from the viewpoint of animal welfare besides its periodical updating with the state-of-art in the toxicological sciences. Revision of the Test Guidelines is in progress according to the 3Rs principle, reduction, refinement and replacement. The acute toxicity test and the skin and eye irritation/corrosion tests were assumed to be the most problematic ones among the animal tests in animal welfare aspect. Three different test methods have been adopted for the alternative to acute oral toxicity test (Test Guideline (TG) 401), namely, the fixed dose procedure (TG420), acute toxic class method (TG423) and up-and-down procedure (TG425), and the reduction of animals was accomplished. Then the traditional acute oral toxicity test (TG401) has been deleted from the Guidelines. Procedures for irritation/corrosion tests for skin (TG404) and eye (TG405) were reorganized into tier-test system in order to prevent any corrosion or strong irritation to take place. The tier system consists of survey of toxicities of the test chemical, structure-activity relationship, pH, and testing with in vitro methods. Moreover, at the final tier animal testing should proceed by one animal. Three kinds of in vitro corrosivity tests have been adopted in the Guidelines.

Keywords: toxicity tests, alternative methods, guidelines, validation

緒　　言

試験法ガイドラインは，試験法を標準化し，一定の技術的な水準を確保し，試験の結果を必要な規制に利用するために設定されるものである．国際的な共通試験法ガイドラインは，試験対象物の国際的流通を促進するために必要とされる．ガイドラインの技術的な側面については，各関係国の科学技術の水準が問われるのみであるが，化学物質規制や安全性評価のような領域に立ち入るときには，各国の政治的状況，国家的，民族的なものの考え方，生活慣習にも及ぶので，科学を超えた作業となる．安全性試験における動物福祉の問題はまさにそのような課題である．しかし，化学物質の安全性評価という作業は，人の生活様式に無関係ではありえず，ガイドラインの位置というものも，科学技術にのみ固執してはいられないものである．安全性試験が動物実験を中心としている限り，提起されている動物福祉の課題に十分に応えなければならない．このたび日本環境変異原学会と日本動物実験代替法学会との合同学術大会での「動物福祉と安全性試験」と題するシンポジウムにおいてOECDガイド

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受付: 2005年5月13日　受理: 2005年5月13日

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本稿は第33回日本環境変異原学会，第18回日本動物実験代替法学会合同学術会議，JEMS & JSAAE合同シンポジウム3「動物福祉と安全性試験」で発表された．
This paper was presented to the JEMS & JSAAE combination symposium 3 "Animal welfare and biosafety tests" at the 33rd JEMS annual meeting and the 18th JSAAE annual meeting, 2004.

ラインにおける動物福祉の取り組みを紹介する機会を与えられたので，その概要を述べる．

1．OECD試験法ガイドラインの成立

OECD(Organisation for Economic Cooperation and Development，経済協力開発機構)とは，元来経済活動のための国際機関として結成されたが，化学物質の環境への影響が重大な問題であることを認め，1970年環境局を設置した．当時大きな問題であったのはPCBの環境汚染であり，日本を含め各国が化学物質規制に踏み出す動きを始めていた．わが国の化学物質審査規制法(「化審法」)は1975年に成立し，各国は相次いで同様の規制を開始した．OECDでは化学物質試験法の国際共通化が必要であると認め，1977年に化学物質試験法ガイドライン計画(Test Guidelines Programme)を開始した．OECDの加盟国は当時23カ国であったが，当時の「西側」の主要先進工業国を網羅しており，国際的な化学物質管理には適切な機関であった．全加盟国の代表による作業の結果，1981年に「OECD化学物質試験法ガイドライン集(Guidelines for Testing of Chemicals)」が成立した．

このガイドラインは，全加盟国の合意を得て成立したharmonizeされた試験法であり，各国は基本的にこのガイドラインに従ったそれぞれの国の規制によって管理を行うが，本質的にこれに従っている限り加盟国間では試験データの相互受け入れ(Mutual Acceptance of Data)の原則があるため，試験された化学物質の輸入に際し試験を反復する必要はない．これは，試験に用いる資源の節約，とくに実験動物の使用数を削減する効果がある．

2．OECDガイドラインの概要

OECD化学物質試験法ガイドラインは次の4部に分かれている．

1) 物理・化学的性質の試験 Physical-Chemical Properties(22試験)
2) 生態系への影響の試験 Effects on Biotic Systems (23試験)
3) 分解性・蓄積性の試験 Degradation and Accumulation(14試験)
4) 健康への影響の試験(毒性試験)Health Effects(51試験)

括弧内の試験法の数は，2004年末の数字である．第4部の健康影響試験は1981年の成立当初は17試験であったが，1983年にAmes試験を含む一群の遺伝毒性試験が加えられるなど，次々に試験法が追加され，現在51試験法を数えている．そのリストをTable 1に示す．実験動物を用いる試験はすべて第4部に含まれ，51のうち38が動物試験で，Table 2に示すとおりである．このほかの動物試験としては，主に生態系への影響の試験として，鳥，魚，昆虫(ショウジョウバエ，ミツバチ)，ミジンコ，ミミズを用いるものがあるが，ほとんど動物福祉の関心が及ばないものである．動物試験については，3Rの原則(Reduction, Refinement, Replacement)に基づく代替法の開発が種々試みられている．なお，第4部の試験は400番台の数字で整理されている．

3．試験法ガイドライン計画の展開

OECD試験法ガイドライン集は1981年に完成してから後，定期的な見直しを行って技術的な水準を維持するよう努める方針が講じられたが，高次会議からの指令によって動物福祉の観点からの見直しも行うようになった．急性経口毒性試験と皮膚および眼の刺激性/腐食性試験について，応急的な改定が加えられ，同時に代替法の開発研究が行われた．In vitro試験法による代替法開発の傍ら，新しい試験法の有用性の検証(validation)の手順が考慮された．急性経口毒性試験(Test guideline (TG)401)の代替法としてTG420，TG423，TG425の3試験が提案され，validationを経て採択された．この問題に関連して指針文書(Guidance Document, GD)「急性毒性試験の指針」(GD24)が編纂され，「人道的方法の指針」(GD19)もまとめられた．

試験法ガイドライン計画は，OECDに事務局を置き，各加盟国の調整員(National Coordinators, NC)が自国内の行政，専門家，関連業界等と調整を取りながら，連絡を取り合い，年1回程度会合してガイドライン作成・改定作業を進めている．NC会議の結果は，上位の行政官の会議であるJoint Meetingに報告され，そこで合意されたものが最終的に理事会で承認されて決定する．試験法ガイドラインの技術的部分については必要に応じ専門家の会議に諮問をして討議を尽くす．NCの会議には，企業団体の代表や，動物愛護団体協議会の代表(ICAPO)も出席して意見を述べている．

4．急性毒性試験代替法の採用とLD_{50}試験の廃止

数ある動物試験の中で，急性毒性試験が動物福祉の観点から改定が必要と見なされたのには複雑な経緯があるが，多数の動物をLD_{50}の数値を求めるために消費するという点に最も大きな批判があった．LD_{50}は変動のある生物反応の推計値であり，測定条件によっても変動する数値であることから，価値が低いとされた．そこでLD_{50}値のより正確に推定しようとすることを止め，使用動物数を削減する方法が考案された．固定用量法(TG420)，急性毒性等級法(TG423)および上げ下げ法(TG425)である．TG423は3匹ずつ，TG425は1匹ずつの試験を一定の手順ですすめ，最終的には動物の死亡を指標にして急性毒性を評価する方法であるが，TG420は，動物は死亡に至らないが確実な毒性徴候を示す用量を求める方法で，理想的に進行すれば動物を死に至らせるこ

Table 1 List of the OECD guidelines for testing health effects

401 急性経口毒性 Acute Oral Toxicity (Deleted 20 Dec 2002)
402 急性経皮毒性 Acute Dermal Toxicity
403 急性吸入毒性 Acute Inhalation Toxicity
404 急性皮膚刺激性/腐食性 Acute Dermal Irritation/Corrosion
405 急性眼刺激性/腐食性 Acute Eye Irritation/Corrosion
406 皮膚感作性 Skin Sensitisation
407 嚙歯類28日反復経口投与毒性試験 Repeated Dose 28-day Oral Toxicity Study in Rodents
408 嚙歯類90日反復経口投与毒性試験 Repeated Dose 90-day Oral Toxicity Study in Rodents
409 非嚙歯類90日反復経口投与試験 Repeated Dose 90-day Oral Toxicity Study in Non-Rodents
410 21/28日反復経皮投与毒性試験 Repeated Dose Dermal Toxicity: 21/28-day Study
411 亜慢性皮膚毒性：90日試験 Subchronic Dermal Toxicity: 90-d Study
412 反復投与吸入毒性：28日/14日試験 Repeated Dose Inhalation Toxicity: 28-day or 14-day Study
413 亜慢性吸入毒性：90日試験 Subchronic Inhalation Toxicity: 90-day Study
414 出生前発生毒性試験 Prenatal Developmental Toxicity Study
415 一世代繁殖毒性試験 One-Generation Reproduction Toxicity Study
416 二世代繁殖毒性試験 Two-Generation Reproduction Toxicity Study
417 トキシコキネティクス Toxicokinetics
418 有機リン化合物急性曝露後の遅発性神経毒性 Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure
419 有機リン化合物遅発性神経毒性：28日反復投与試験 Delayed Neurotoxicity of Organophosphorus Substances: 28-day Repeated Dose Study
420 急性経口毒性－固定用量法 Acute Oral Toxicity—Fixed Dose Method
421 簡易生殖発生毒性試験 Reproduction/Developmental Toxicity Screening Test
422 反復投与毒性生殖発生毒性併合試験 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test
423 急性経口毒性－急性毒性等級法 Acute Oral Toxicity—Acute Toxic Class Method
424 嚙歯類神経毒性試験 Neurotoxicity Study in Rodents
425 急性経口毒性－上げ下げ法 Acute Oral Toxicity: Up-and-Down Procedure
426 発生神経毒性試験 Developmental Neurotoxicity Study
427 In vivo 経皮吸収試験 Skin absorption: In Vivo Method
428 In vitro 経皮吸収試験 Skin absorption: In Vitro Method
429 皮膚感作性：局所リンパ節試験 Skin Sensitisation: Local Lymph Node Assay
430 In vitro 皮膚腐食性：経皮電気抵抗試験 In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)
431 In vitro 皮膚腐食性：ヒト皮膚モデル試験 In Vitro Skin Corrosion: Human Skin Model Test
432 In vitro 光毒性試験 In Vitro 3T3 NRU Phototoxicity Test
435 In vitro 皮膚腐食性：膜障壁試験 Membrane Barrier Test
451 癌原性試験 Carcinogenicity Studies
452 慢性毒性試験 Chronic Toxicity Studies
453 慢性毒性癌原性併合試験 Combined Chronic Toxicity/Carcinogenicity Studies
471 細菌による復帰突然変異試験 Bacterial Reverse Mutation Test
473 培養細胞染色体異常試験 In vitro Mammalian Chromosomal Aberration Test
474 赤血球小核試験 Mammalian Erythrocyte Micronucleus Test
475 骨髄細胞小核試験 Mammalian Bone Marrow Chromosomal Aberration Test
476 培養細胞遺伝子突然変異試験 In vitro Mammalian Cell Gene Mutation Test
477 ショウジョウバエ伴性劣性致死試験 Sex-Linked Recessive Lethal Test in *Drosophila melanogaster*
478 嚙歯類優性致死試験 Rodent Dominant Lethal Test
479 培養細胞姉妹染色分体交換試験 In vitro Sister Chromatid Exchange Assay in Mammalian Cells
480 酵母遺伝子突然変異試験 *Saccharomyces cerevisiae*, Gene Mutation Assay
481 酵母体細胞組換え試験 *Saccharomyces cerevisiae*, Mitotic Recombination Assay
482 培養細胞DNA傷害修復，不定期DNA合成試験 DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells in vitro
483 精原細胞染色体異常試験 Mammalian Spermatogonial Chromosome Aberration Test
484 マウススポットテスト Mouse Spot Test
485 遺伝性転座試験 Mouse Heritable Translocation Assay
486 肝細胞不定期DNA合成試験 Unscheduled DNA Synthesis Test with Mammalian Liver Cells in vivo

Table 2　Whole animal experiments in the OECD test guidelines

急性毒性試験	● 401 経口，● 402 経皮，● 403 吸入，◎ 420, 423, 425 経口
反復投与毒性	● 407 28日経口，● 408 90日嚙歯類，● 409 90日非嚙歯類， ● 410 21日経皮，● 411 90日経皮，● 412, 413 反復吸入
長期毒性試験	● 451 癌原性，● 452 慢性毒性，● 453 慢性・癌原性併合
遺伝毒性試験	● 474 小核試験，● 478 優性致死，● 483 精原細胞染色体， ● 484 マウススポット，● 485 遺伝性転座，● 486 肝UDS， ○ 477 ショウジョウバエ
生殖発生毒性	● 414 出生前発生毒，● 415 一世代繁殖，● 416 二世代繁殖， ● 421 簡易生殖発生，● 422 反復投与毒性/生殖発生併合
刺激性/腐食性	◎ 404 皮膚，◎ 405 眼
免疫毒性/感作性	● 406 皮膚感作性，● 429 局所リンパ節
薬物/毒物代謝	● 417 トキシコキネティクス，● 427 In vivo 経皮吸収
神経毒性試験	● 424 嚙歯類神経毒性，● 426 発生神経毒性， ○ 418 遅発神経毒性:急性曝露，○ 419 遅発神経毒性：28日反復曝露
生態系毒性試験	○ 205, 206, 223 鳥，○ 203, 210, 212, 215 魚，○ 207 ミミズ， ○ 213, 214 ミツバチ ○202, 211 ミジンコ

● 哺乳類を用いる試験，◎ 改良済試験，○非哺乳類動物を用いる試験

Table 3　Numbers of animals used in acute oral toxicity tests

試験法		条　件	動物数
・TG 401	原法（1981年）	1群5匹，両性，5群～	50～100～
	改法（1987年）	1群5匹，片性，5群～	[10] 20～40
・TG 420	原法（1992年）	1群5匹，両性，1～2用量	[10] 14～24
	改法（2001年）	1群5匹，片性，1～2用量	[5] 6～20
・TG 423	原法（1996年）	1群3匹，両性，2用量	[6] 12～24
	改法（2001年）	1群6匹，片性，2用量	[6] 12～24
・TG 425	原法（1998年）	逐次1匹，片性，f＝1.3	[6] 8～10 (18～30)
	改法（2001年）	逐次1匹，片性，f＝3.2	[6] 8～15

[ ]：最小

となく急性毒性を評価するものである．ただし，どの方法にも，動物が苦痛を示す状況では試験を中断して動物を安楽死させるべしとの規定がある．これらの方法によって，使用動物数は大幅に削減でき，動物の苦痛も最少に止める効果が期待される(Table 3)．

動物の苦痛については，人により，学問の流派によって，また国家民族的習慣によって，理解の相違がある．試験を中断して安楽死させるということは，試験の目的達成を阻害することであるから，その決断は難しい．そこで，何が人道的な方法かを示す基準をOECDはまとめて，指針として発行した．「実験動物の臨床症状の認識，評価，利用の指針：安全性試験における人道的判断の基準」(指針文書No.19)である．

本来代替法というものは，本試験法と同等の有用性をもつので代替可能な方法として採択されるものであり，本試験を排除し置換するものではない．しかし，この急性毒性試験の場合は，TG401は数年に及ぶ議論の末，廃棄されることとなった．採択された代替法が実際として利用されず，依然として伝統的なTG401による急性毒性試験が行われていたからである．2001年12月17日TG401の削除が合意され，1年後の2002年12月20日以降行われたTG401による試験データは相互受け入れの対象にはならないこととされた．

5．皮膚・眼刺激性/腐食性試験の動物福祉対応

皮膚刺激性/腐食性試験(TG404)と眼刺激性/腐食性試験(TG405)もまた動物福祉上問題が多いとされた試験であり，摘出器官での試験から下等動物を用いる方法や細胞毒性試験や非生物モデルによる試験に至る各種の代替法が考案されたが，本試験を代替する十分な試験法は未だ開発されていない．そこで，OECD試験法では，段階的試験手順を取り入れ，最終的には動物で刺激性がないか，あっても軽度であることの確認を行う総合的試験進行方式を採用した．すなわち動物試験に進む前に被験物質の物性，毒性の検討やin vitro 試験法によって調査して，強い刺激性や腐食性の予想されるものは動物試験に進まずに強刺激性または腐食性の物質であると判断しておくという方策である．動物での試験もまず1匹について試験し，何も異常がないときに動物を追加して計3匹で確認するのである．

試験の早い段階で腐食性の(疑いの)有無を検査するin vitro皮膚腐食性試験法がvalidationを経て採択されている．ラットの摘出皮膚を用いる「経皮電気抵抗試験 Transcutaneous Electrical Resistance」(TG430)，皮膚細

胞の三次元培養による再構築を行った「ヒト皮膚モデル試験 Human Skin Model Test」(TG431)，および人工膜の破壊性を検査する「膜障壁試験 Membrane Barrier Test」(TG435)の3種である．

6．代替試験法の検証 (validation) の手順の確立

動物実験代替法の研究の過程において，試験法の validation の方法が確立されてきたことは大きな収穫であった．それは，試験とは何を目的とし，何をもって達成とするかを意識して論議し，信頼性のある試験法であることの基準を明らかにした．その手順では，十分な数の試験物質について一定の手順に従って試験するとき，複数の試験施設からの結果が，どの程度一致するか，再現性があるかを客観的に評価するものである．OECD は1990年に John Frazier に委嘱して「In vitro 毒性試験法の有用性検証の科学的基準」のモノグラフを作成，発行した．この基準は，その後欧州，米国で検討され，1996年 Solna で開かれた OECD ワークショップでまとめられた．さらに2002年に Stockholm で follow-up の会議が催され，試験法検証の手順が確立された．

この手順，少なくともその考え方は，動物実験代替法にとどまらず，新しい毒性試験法の有用性の検証に適用されるべきものであり，内分泌攪乱物質の試験やトキシコゲノミクス応用試験などでこの考え方は生かされるべきであろう．

7．OECD 試験法計画の新しい状況

既存の実験動物を用いる毒性試験法の改良，置換を目指してきた動物福祉対策に今新しい局面が訪れている．それは，さらに大量の毒性試験が新たに要求されているという状況である．OECD ガイドラインが最初に作られたころの化学物質環境汚染問題はわが国の化審法のような各国における化学物質規制に進展したが，当時すでに使用されていた数万に及ぶ「既存化学物質」の安全性点検作業が必要である．10年ほど前に提起された内分泌攪乱物質の試験法の確立と，多数の物質についての試験が控えている．小児の健康への化学物質の影響が懸念されており，新しい試験法も含め，どの程度の試験が必要かまだ不明であるが，相当の量の動物試験が行われることは必須である．そうした，人類の生存と福祉 well-being と動物福祉をどのように調和させていくかがこれからの課題であろう．

The Journal of Toxicological Sciences,
Vol.30, No.3, 175-194, 2005

INFLUENCE OF DI-(2-ETHYLHEXYL)PHTHALATE ON FETAL TESTICULAR DEVELOPMENT BY ORAL ADMINISTRATION TO PREGNANT RATS

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(Received January 18, 2005; Accepted April 8, 2005)

ABSTRACT — Influence of di-(2-ethylhexyl)phthalate (DEHP) on testicular development was studied by oral administration of DEHP at doses of 500 and 1000 mg/kg/day to pregnant rats on gestational days (G) 7 to 18. Ethinyl estradiol (EE) at dose levels of 0.25 and 0.5 mg/kg/day was used as a reference substance. Each 5-6 pregnant rats were sacrificed and their fetuses were examined on G12, 14, 16, 18 and 20. Fetal deaths averaging 20-36% were observed at every examination in the group receiving 1000 mg/kg of DEHP. Increases of fetal deaths over 50% were also observed in the reference group that received 0.5 mg/kg of EE. Microscopic examination of the fetal testis in groups treated with DEHP revealed degeneration of germ cells in G16 fetuses and localized proliferation or hyperplasia of interstitial cells in G18 and 20 fetuses. Germ cells having more than two nuclei were observed in a few cases including the control testes of G14 fetuses. These multinucleated cells were observed frequently in G20 fetuses treated with DEHP. Examination of testes of naturally delivered offspring of dams treated with 1000 mg/kg of DEHP at 7 weeks of age revealed scattered atrophy or dilatation of seminiferous tubules.

Another experiment was carried out to confirm the dose of DEHP affecting testicular development and spermatogenesis. DEHP was given to pregnant rats at doses of 125, 250 and 500 mg/kg/day during G7-18. Similar histopathological changes were observed in fetal testes of the group exposed to 500 and 250 mg/kg of DEHP, but not in those exposed to 125 mg/kg. In postnatal examinations, however, no abnormality was found in the testes at 5 and 10 weeks after birth in any of the treated groups. Furthermore, no abnormal findings were observed in the function of sperm, sperm counts and sperm morphology in the offspring of the group treated with DEHP during the fetal period at 10 weeks of age. Thus, 125 mg/kg/day is considered the no-observed-effect-level of DEHP on testicular development of rats by exposure *in utero* during the period of organogenesis.

KEY WORDS: Phthalic acid ester, Developmental toxicity, Testicular toxicity, Sertoli cells, Sperm function, Rats

INTRODUCTION

It has been shown that high doses of phthalic acid esters exert testicular toxicity in animals (Calley *et al.*, 1966; Gangolli, 1982). Toxic effects on the testis were similarly observed with a variety of phthalate esters such as di-(2-ethylhexyl) phthalate (DEHP) (Gray *et al.*, 1977), dibutylphthalate, (Cater *et al.*, 1977) and di-n-pentylphthalate (Creasy *et al.*, 1983, 1987). Among a variety of phthalate esters, DEHP has been investigated most frequently as a representative substance of phthalic acid esters. The mechanism of the testicular toxicity of phthalates is not yet wholly clear, although the damaging effect on Sertoli cells and blood-testis

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barrier has been considered (Gray and Butterworth, 1980). We have conducted a series of experiments on testicular toxicity of DEHP in rats, and have clarified that ultrastructural changes were induced in seminiferous tubules at stages from IX to I of the spermatogenic cycle in 8 week-old Sprague-Dawley rats, 3 to 18 hr after single-dose administration of 2,800 mg/kg of DEHP (Saitoh *et al.*, 1997). Noteworthy changes were degeneration of spermatocytes, dilatation of rough-surfaced endoplasmic reticulum, especially those in the vicinity of the tight junction of ectoplasmic specialization of Sertoli cells, and disintegration of the intercellular junction between Sertoli cells. In a study utilizing electron microscopic autoradiography, we have demonstrated the distribution of phthalic acid into the testis, especially to Sertoli cells (Ono *et al.*, 2004). We have also observed that clear structural changes of testes were induced with single oral dose of 1400 mg/kg, and that the non-toxic dose level of DEHP on testes was 700 mg/kg in mature rats. Furthermore, we have employed a lanthanum trace method to examine the effects of DEHP on Sertoli cell function, especially on the condition of blood-testis barrier in rats (Saitoh *et al.*, 1997). In this study, lanthanum particles were observed 6 hr after administration at the tight junction between Sertoli cells, which showed that the function of Sertoli cells to maintain the blood-testis-barrier was affected with DEHP as early as 6 hr after oral administration, but had recovered by 24 hr. The fetal stage is known to be vulnerable to chemical exposure, and the effects on gonadal and endocrine systems are of special concern. In this context, de Kretser and Kerr (1994) described that the blood-testis barrier in rats was established during 16~19 days of postnatal life. In the present study, influence of *in utero* exposure to DEHP on development of testes in rats was examined. Ethinyl estradiol was used as a reference substance for estrogenic activity of DEHP, if any.

MATERIALS AND METHODS

Materials

Di-(2-ethylhexyl)phthalate (DEHP) was purchased from Wako Pure Chemical Industries Ltd. and was diluted with corn oil (Nacalai Tesque Inc.) to a concentration appropriate for administration at the constant volume of 5 mL/kg. Ethinyl estradiol (EE, Wako Pure Chemical) was suspended in corn oil on the same principle and used as the reference compound.

Animals

Adult rats of Sprague-Dawley strain (Crj: CD IGS) were purchased from Charles River Japan Inc., and were kept for a week to acclimatize them to the laboratory condition. The animals were reared individually in a metallic cage sized 220×270×190 mm, in a room with conditioned temperature at 24~26°C and relative humidity within 50~65%. Lighting was alternated at 12 hr intervals (lights on 7:00~19:00). Appropriate bedding material such as White flake (Charles River) was provided for pregnant and nursing rats. The animals were fed with pellet food (CE-2, CLEA Japan Inc.) *ad libitum* and were supplied with tap water.

A female rat was mated with a male and a vaginal smear specimen was examined every morning. The day when a vaginal plug or sperm in the specimen was confirmed was defined as gestational day (G) 0. The pregnant animals were allocated to groups in a random fashion stratified by body weight on the day of administration (G7).

Dosage and administration

Preliminary dose-finding study showed that administration of 2000 mg/kg/day DEHP to pregnant rats from G7 to G18 caused high incidence of absorption of embryos and fetal deaths. Similar administration of 1000 mg/kg/day of DEHP caused a few fetal deaths and some pathological findings in the testis. Thus the doses of DEHP were decided on 1000 mg/kg for the highest and 500 mg/kg for the lowest in the first experiment. The doses of DEHP in Experiment 2 were selected to be 500, 250 and 125 mg/kg, considering the results of the first experiment. The doses of EE were set at 0.5 and 0.25 mg/kg referring to the study by Yasuda *et al.* (1985) in mice. Oral administration by gavage was started on G7 and continued till G18.

Experimental design

The study was designed in two phases; observation of the histopathological changes of testicular development by intra-uterine exposure to DEHP was made in Experiment 1, including the dose finding, and in Experiment 2 a search for the no-effect level was attempted, together with confirmation of the findings in Experiment 1. .

In Experiment 1, 28-30 dams per group were given oral administration of DEHP, EE or the vehicle from G7 to G18. Each 6 of these dams were killed by ether inhalation on G12, 14, 16, 18 and 20 to examine their fetuses. In addition, each 5 dams of groups given 500 and 1000 mg/kg of DEHP were allowed to deliver

spontaneously to examine postnatal changes in the testis and epididymis of their offspring. The male offspring were reared and kept until examination at 7 weeks of age.

In Experiment 2, each 11-12 pregnant females were given oral administration of DEHP or vehicle. Each 3 dams of the groups were submitted to Caesarean section on G20 to examine their fetuses. Other dams were allowed to deliver spontaneously and male offspring chosen for examination at 5 and 10 weeks of age. The day of delivery was defined as Day 0 of lactation.

Observations of dams

Dams were examined daily for general conditions in all experiments and body weight was measured occasionally. Delivery and nursing conditions were observed and the numbers of fetuses delivered and live offspring were determined. From these data and the number of implantations counted at the necropsy, viability of the offspring, namely, delivery index (fetuses delivered/implantation sites, %), birth index (live offspring at birth/implantation sites, %), viability index (live offspring on day 4 of lactation/live offspring at birth, %) and weaning index (live offspring on day 21 of lactation/live offspring on day 4 of lactation, %), were determined.

Examination of the fetuses and offspring

In Experiment 1, fetuses on G12 were collected only for histopathological examination. Live fetuses collected on G14, 16, 18 and 20 were weighed and the external appearances examined. Whole bodies and testes from these live fetuses were submitted for histopathological or electron microscopic examination. The testes and epididymides of male offspring of the DEHP-treated groups were collected at 7 weeks of age for histopathological examination.

For histopathological examination, the specimens were fixated in Bouin's solution and then immersed in a buffered neutral formalin solution. The fixed tissues were embedded in paraffin and cut in 4 μm slices. These sections were stained with hematoxylin and eosin (HE) and were examined under light microscopy.

For electron microscopic examination, the tissues were immersed in an ice-cold mixture of 2% paraformaldehyde buffered with 0.1 M s-collidine and 1.25% glutaraldehyde for 3 hr. The fixed tissues were cut into small pieces and post-fixed with 2% osmium tetraoxide buffered with 0.1 M s-collidine. The post-fixed tissues were dehydrated in ethanol and embedded in epoxy resin (Quetol-651, Nissin EM, Tokyo). Semithin sections (1 μm) were stained with toluidine blue and observed under a light microscope. Representative areas were selected from the testis preparations and ultra-thin sections were prepared and stained with uranyl acetate and lead citrate, and then examined with an electron microscope (H-7100, Hitachi, Tokyo).

In Experiment 2, all of the live fetuses examined on G20 were weighed by sex and examined for their external appearance, and then testes were dissected from live male fetuses for histopathological examination as described in Experiment 1, and for staining of androgen receptors. The offspring were weighed and reared until examination. Each 4 male offspring from each group were killed at 5 and 10 weeks of age, and testes with epididymides were dissected and HE-stained thin sections prepared as described above. For electron microscopic examination, each 2 male offspring were used and fixation was performed by a systemic perfusion of a mixture solution of 2% paraformaldehyde buffered with 0.1 M s-collidine and 2.5% glutaraldehyde from the aorta to the body with a perfusion pump under sodium pentobarbital anesthesia. The testes were submitted to electron microscopic observation. The other 4 offspring of each group were killed by ether inhalation at 10 weeks of age to obtain their testes and epididymides for sperm examination.

Immunohistochemistry of androgen receptors

In addition, in order to confirm the development of hormone receptors, expression of androgen receptors in the testis was observed by an immunohistochemical method (Dalgaard *et al.*, 2001), using a rabbit polyclonal antibody for N-terminal of the androgen receptor (AR-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Examination of spermatogenesis

In Experiment 2 the seminiferous epithelium cycle was examined on testis sections stained with HE obtained at 5 weeks of age, and the spermatogenic stage was determined according to the simplified method described by Matsui *et al.* (1996). Briefly, seminiferous tubules on a specimen were classified into four groups by spermatogenic stages I-VI, VII-VIII, IX-XI and XII-XIV (Dym and Clermont, 1970). One corresponding section of the testis was stained with periodic acid Schiff (PAS) to confirm acrosomes of spermatogonia. Each 5 seminiferous tubules belonging to 4 groups were chosen and the numbers of germ

cells and Sertoli cells in a tubule determined to calculate a ratio of germ cells to Sertoli cells in each group.

Analysis of morphology and function of sperm

Sperm were collected through micropuncture of the cauda epididymis of rats at 10 weeks of age, and were examined as previously described (Sato *et al.*, 2000). Sperm motility was measured using a computer-assisted sperm motion analysis system (HTM-IVOS ver 10.6, Hamilton-Thorne Research, Beverly, MA, USA) and for morphological analysis of spermatozoa as described previously (Sato *et al.*, 2002a). After the collection of sperm for motility analysis, the cauda epididymis was dissected at the transition point to the vas deferens and at the middle of the cauda and body of epididymis, weighed and then stored at –20°C. The frozen cauda epididymis was thawed to room temperature and homogenized in distilled water. The sperm heads in the homogenate were counted with HTM-IVOS as previously described (Sato *et al.*, 2002b).

Statistical analysis

When uniformity of variance was confirmed among the groups by the method of Bartlett, data obtained were analyzed by ANOVA (Yoshimura, 1986). When the uniformity was not confirmed, Kruskal-Wallis's rank-sum test was applied instead (Yoshimura, 1986). When significant differences between groups were observed in either of the analyses, Dunnett test was applied for a comparison between the control and treated groups of either DEHP or EE (Yoshimura, 1986). A p value less than 0.05 was considered statistically significant.

RESULTS

Effects of DEHP treatment on dams

Daily oral administration of DEHP at a level of 1000 mg/kg and EE at levels of 0.25 and 0.5 mg/kg slightly suppressed body weight gain of pregnant rats during the treatment period. Administration of the lower dose levels of DEHP did not affect maternal body weight (Tables 1 and 2).

Effects of maternal treatment with DEHP on fetuses and offspring

Reproductive performance data, including fetal weights on G14, 16, 18 and 20 in Experiment 1, are summarized in Table 1. Oral DEHP treatment at 1000 mg/kg reduced fetal body weights at G14 and 18 significantly ($p<0.01$) as compared with those of the control group. Furthermore, 1000 mg/kg of DEHP treatment increased intrauterine mortality to 20-36%. DEHP treatment at 500 mg/kg did not cause increase in fetal deaths and changes in fetal body weight significantly. Treatment with 0.5 mg/kg of EE also increased intrauterine mortality of fetuses, even to more than 50% on G16 and 20.

External observation of fetuses on G20 revealed various malformations in treated groups. Two fetuses with branchyury from a single dam given 500 mg/kg DEHP were observed and each one fetus with general edema, club foot or anal atresia and 3 fetuses with kinked tail from a single dam given 1000 mg/kg of DEHP were observed. In the group treated with 0.5 mg/kg of EE, one fetus with kinked tail was observed. Two out of 5 dams given 500 mg/kg DEHP did not deliver any offspring because of early embryonic loss. However, 1000 mg/kg of DEHP did not cause any abnormality in delivery.

In Experiment 2, DEHP-treatment up to 500 mg/kg did not show any marked effect on fetuses (Table 2). External malformations observed in the 500 mg/kg group in Experiment 1 were not reproduced in Experiment 2. Birth weights of the offspring were significantly higher in the groups exposed to DEHP at 250 and 500 mg/kg than control. Viability and growth rate of the offspring are summarized in Table 3. Differences of body weight among the groups were insignificant on the 4th day of lactation.

Histopathological findings of fetuses and offspring

Histopathological findings of fetal testes in Experiment 1 are summarized in Table 4. Representative photographs are shown in Photos 1~3. The testis was not distinguishable in the fetuses on G12, when a few round germ cells with clear cytoplasm were scattered in mesenchyma around mesonephros. The testis was distinguished morphologically on G14, when the germinal ridge was formed and a few germ cells, some showing mitosis, were seen in the gonadal cord. On G16, the testicular cord became prominent, containing many round nucleated germ cells and Sertoli cells on its margin (Photo 1a). On G18, the interstitium was widened in the center of the gonad containing rich interstitial cells (Photo 2a), when the density of germ cells in the reproductive tract was increased. On G20, the testicular cord developed further, although the tubular structure was not yet formed (Photo 3a).

No abnormalities were observed in any group on G14. On G16, degeneration of germ cells was noted in

Table 1. Viability and development of fetuses exposed to di-(2-ethylhexyl)phthalate (DEHP) or ethinyl estradiol (EE) during gestational days 7-18 (Experiment 1).

	DEHP (mg/kg)			EE (mg/kg)	
	0 [a]	500	1000	0.25	0.5
Gestational day 14	(6)	(6)	(6)	(5)	(5)
Maternal body weight (g)	336.9 ± 17.4	326.5 ± 31.0	320.2 ± 14.9**	295.7 ± 14.7**	278.4 ± 22.7**
Implantations	17.0 ± 1.4	15.0 ± 1.7	16.2 ± 1.2	15.6 ± 1.1	13.6 ± 4.7
Intrauterine mortality (%)	7.1 ± 5.9	3.0 ± 5.0	20.3 ± 18.4	3.8 ± 5.6	6.9 ± 9.4
Live fetuses	15.8 ± 2.1	14.5 ± 1.2	12.8 ± 2.9	15.0 ± 1.4	12.6 ± 4.5
Mean fetal weight (g)	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.02**	0.15 ± 0.01	0.15 ± 0.02
Gestational day 16	(5)	(6)	(6)	(0)	(5)
Maternal body weight (g)	344.9 ± 4.9	344.3 ± 21.3	311.4 ± 20.0**		285.7 ± 30.4**
Implantations	15.4 ± 1.3	16.0 ± 1.3	15.3 ± 1.6		13.6 ± 6.2
Intrauterine mortality (%)	1.3 ± 2.8	12.4 ± 7.6	33.1 ± 31.3		72.0 ± 36.9
Live fetuses	15.2 ± 1.3	14.0 ± 1.4	10.2 ± 4.8		4.2 ± 5.4
Mean fetal weight (g)	0.44 ± 0.02	0.43 ± 0.02	0.37 ± 0.04		0.42 ± 0.02 [b]
Gestational day 18	(6)	(6)	(6)	(4)	(5)
Maternal body weight (g)	370.7 ± 36.5	360.0 ± 36.6	335.5 ± 20.2*	327.6 ± 42.4*	321.7 ± 16.3**
Implantations	14.5 ± 1.4	15.2 ± 2.6	14.8 ± 1.6	15.3 ± 2.2	14.4 ± 2.4
Intrauterine mortality (%)	3.6 ± 6.3	1.0 ± 2.4	35.6 ± 36,5	5.7 ± 7.9	1.3 ± 3.0
Live fetuses	14.0 ± 1.8	15.0 ± 2.5	9.5 ± 5.6	14.3 ± 1.0	14.2 ± 2.4
Mean fetal weight (g)	1.35 ± 0.07	1.32 ± 0.06	1.03 ± 0.13**	1.33 ± 0.05	1.25 ± 0.10
Gestational day 20	(6)	(6)	(6)	(0)	(2)
Maternal body weight (g)	404.2 ± 6.5	410.8 ± 30.2	365.0 ± 25.4**		322.8
Implantations	14.7 ± 1.6	14.8 ± 2.6	14.5 ± 1.5		15.5
Intrauterine mortality (%)	0.0 ± 0.0	2.7 ± 4.4	36.4 ± 26.5		50.8
Live fetuses	14.7 ± 1.6	14.5 ± 2.9	9.0 ± 3.5		7.5
Mean fetal weight (g)	3.68 ± 0.20	3.52 ± 0.14	2.82 ± 0.11		2.90
External malformations	0.0 ± 0.0	2.22 ± 5.44	6.25 ± 15.31		7.14

[a] Vehicle control (corn oil, 5 mL/kg). [b] Data from 3 dams having live fetuses.
* Significantly different from control ($p<0.05$). ** Significantly different from control ($p<0.01$).

Table 2. Reproductive parameters on gestational day 20 in rats treated with di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Gestational day 20				
Dams examined	3	3	3	3
Maternal body weight (g)	408.3 ± 32.6	428.8 ± 42.5	399.3 ± 43.4	427.9 ± 50.8
Implantations	14.7 ± 0.6	15.0 ± 2.6	14.0 ± 1.7	16.0 ± 1.7
Intrauterine mortality (%)	2.2 ± 3.9	0	2.8 ± 4.8	4.1 ± 3.6
Live fetuses	14.3 ± 0.6	15.0 ± 2.6	13.7 ± 2.3	15.3 ± 1.5
Males	5.3 ± 1.2	7.0 ± 3.5	6.3 ± 2.1	9.3 ± 2.1
Females	9.0 ± 1.0	8.0 ± 1.0	7.3 ± 0.6	6.0 ± 1.0
Sex ratio (%)	37.2 ± 7.6	44.7 ± 17.2	45.5 ± 8.5	60.5 ± 9.1
Fetal body weight (g)	14.0 ± 1.8	15.0 ± 2.5	9.5 ± 5.6	14.2 ± 2.4
Males	3.77 ± 0.13	3.86 ± 0.40	4.02 ± 0.13	3.57 ± 0.14
Females	3.51 ± 0.14	3.67 ± 0.34	3.77 ± 0.16	3.40 ± 0.03
External malformations	0	0	0	0

Values represent mean ± S.D.
[a] Vehicle control (corn oil, 5 mL/kg).

one of 12 examined fetuses of the 1000 mg/kg DEHP group (Photos 1b, 1c). No such findings were noted in other fetuses of the group exposed to DEHP at 1000 mg/kg and also at 500 mg/kg. On G18, interstitial cells were increased in number and aggregated topically in the 500 mg/kg DEHP group (Photo 2b), and the hyperplasia of interstitial cells was intensified in the 1000 mg/kg DEHP group (Photo 2c), while such findings were not noted in any testes of fetuses exposed to EE. Testicular size was smaller in the groups of 1000 mg/kg DEHP and 0.5 mg/kg EE on G18 and G20. On G20, germ cells having more than two nuclei were noted and thickened seminiferous cords containing rich germ cells were seldom observed in the 500 mg/kg DEHP group. In fetal testes of the 1000 mg/kg DEHP group hyperplasia of interstitial cells, multinucleated germ cells were also seen (Photos 3b, 3c). Topically thickened seminiferous cords due to aggregation of germ cells were observed frequently in this group.

Table 5 summarizes histopathological findings in the testis of the offspring in Experiment 1. Representative pictures are shown in Photos 4~6. In the offspring at 7 weeks after birth prenatally exposed to DEHP at a level of 500 mg/kg, no obvious abnormalities were found except for multinucleated giant cells in the seminiferous tubules and cell debris in the epididymal lumens (Photos 4a, 4b). In the 1000 mg/kg-exposed group, however, most of the animals had developed abnormalities, such as branched seminiferous tubules with atrophy and/or dilatation, multinucleated giant cells and dilatation of rete testis (Photos 4c, 5a, 5b). In addition to these findings, testes from several animals in this group showed hyperplasia of the interstitial cells (Photo 4c), necrosis and/or mineralization of testes, foreign body giant cells, focal loss of seminiferous tubules and malformed seminiferous tubules (Photos

Table 3. Reproductive data and development of the offspring treated with di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Dams examined	8	9	8	8
Gestation length (days)	21.8 ± 0.5	22.0 ± 0.0	22.0 ± 0.0	22.0 ± 0.0
Implantation sites	15.4 ± 1.2	15.6 ± 2.4	15.4 ± 1.1	14.9 ± 1.2
At birth (Day 0 of lactation)				
Live offspring	14.0 ± 2.1	14.6 ± 2.6	14.4 ± 1.7	14.1 ± 1.2
Birth index (%) [b]	90.8 ± 9.0	93.2 ± 6.3	93.5 ± 8.8	95.1 ± 5.7
Sex ratio (%)	42.0 ± 10.4	45.7 ± 8.9	42.9 ± 12.3	50.5 ± 12.3
Body weight, males (g)	6.5 ± 0.3	6.7 ± 0.5	7.0 ± 0.5	7.1 ± 0.3*
Body weight, females (g)	6.1 ± 0.3	6.3 ± 0.5	6.7 ± 0.6*	6.7 ± 0.3*
Day 4 of lactation				
Live offspring	13.9 ± 2.2	14.3 ± 2.5	14.4 ± 1.7	14.0 ± 1.3
Viability (%)	99.0 ± 2.7	98.6 ± 2.8	100.0 ± 0.0	99.1 ± 2.5
Sex ratio (%)	42.3 ± 10.1	46.3 ± 8.5	42.9 ± 12.3	50.9 ± 11.4
Body weight, males (g)	10.3 ± 1.1	10.4 ± 1.0	10.7 ± 0.7	10.5 ± 1.3
Body weight, females (g)	9.8 ± 1.2	9.7 ± 1.0	10.3 ± 0.8	10.0 ± 1.3
Body weight, preserved males (g) [c]	10.4 ± 0.9	10.7 ± 0.7	11.0 ± 0.5	10.7 ± 0.1
Day 7 of lactation				
Body weight, preserved males (g)	17.1 ± 2.3	16.9 ± 1.0	18.2 ± 0.7	17.2 ± 0.2
Day 14 of lactation				
Body weight, preserved males (g)	36.1 ± 2.8	34.5 ± 1.5	37.5 ± 0.8	37.6 ± 1.3
At weaning (Day 21 of lactation)				
Body weight, preserved males (g)	58.7 ± 4.7	57.1 ± 4.1	62.2 ± 1.5	60.5 ± 3.3
Weaning index (%)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Values represent mean ± S.D. * Significantly different from control ($p<0.05$).

[a] Vehicle control (corn oil, 5 mL/kg). [b] Live offspring/implantation sites.

[c] Each 2~3 male offspring from dams were preserved.

Table 4. Histopathological findings of testes of fetuses exposed to di-(2-ethylhexyl) phthalate (DEHP) or ethinyl estradiol (EE) during gestational days 7-18 (Experiment 1).

Group	DEHP 0 mg/kg					DEHP 500 mg/kg					DEHP 1000 mg/kg					EE 0.25 mg/kg					EE 0.5 mg/kg				
Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Gestational day 12	(12)					(10)					(10)										(10)				
Degeneration of fetal tissue	12	0	0	0	0	10	0	0	0	0	8	0	0	2	0						2	0	0	8	0
Gestational day 14	(9)					(9)					(9)					(9)					(9)				
Multinucleated germ cells	8	1	0	0	0	9	0	0	0	0	8	1	0	0	0	9	0	0	0	0	8	1	0	0	0
Gestational day 16	(10)					(10)					(12)										(4)				
Degeneration of germ cells	10	0	0	0	0	10	0	0	0	0	11	0	0	1	0						4	0	0	0	0
Multinucleated germ cells	10	0	0	0	0	9	1	0	0	0	10	2	0	0	0						4	0	0	0	0
Gestational day 18	(20)					(20)					(10)					(11)					(16)				
Multinucleated germ cells	20	0	0	0	0	18	2	0	0	0	10	0	0	0	0	11	0	0	0	0	14	2	0	0	0
Increased germ cells in a cord	20	0	0	0	0	20	0	0	0	0	8	2	0	0	0	11	0	0	0	0	16	0	0	0	0
Hyperplasia of interstitial cells	20	0	0	0	0	8	12	0	0	0	0	3	7	0	0	11	0	0	0	0	16	0	0	0	0
Decrease in testicular size	20	0	0	0	0	20	0	0	0	0	4	0	6	0	0	11	0	0	0	0	9	7	0	0	0
Gestational day 20	(17)					(17)					(18)										(8)				
Multinucleated germ cells	16	1	0	0	0	0	0	10	7	0	0	3	13	2	0						4	4	0	0	0
Increased germ cells in a cord	17	0	0	0	0	14	3	0	0	0	1	12	5	0	0						5	3	0	0	0
Hyperplasia of interstitial cells	17	0	0	0	0	0	0	14	0	0	0	1	17	0	0						8	0	0	0	0
Decrease in testicular size	17	0	0	0	0	17	0	0	0	0	5	8	5	0	0						5	3	0	0	0

− : negative, ± : very slight, + : slight, ++ : moderate, +++ : severe.
Figures in parentheses show number of dams examined.