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Involvement of the Retinoid X Receptor in the Development of Imposex Caused by Organotins in Gastropods

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Organotin compounds released from antifouling paints, such as tributyltin (TBT) and triphenyltin (TPT), are potent inducers of imposex (a superimposition of male genital tracts, such as penis and vas deferens, on females) in marine gastropods. Little is known about the induction mechanism of gastropod imposex. Here, we show that organotins bind the human retinoid X receptors (hRXRs) with high affinity and that injection of 9-cis retinoic acid (RA), the natural ligand of hRXRs, into females of the rock shell (*Thais clavigera*) induces the development of imposex. Cloning of the RXR homologue from *T. clavigera* revealed that the ligand-binding domain of rock shell RXR was very similar to vertebrate RXR and bound to both 9-cis RA and to organotins. These suggest that RXR plays an important role in inducing the development of imposex, namely, the differentiation and growth of male genital tracts in female gastropods.

Introduction

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s and released into the marine environment resulting in a worldwide pollution (*1*). TBT and TPT are very toxic to organisms, including marine species (*2*–*5*). One of the most interesting toxic effects of TBT and TPT to marine organisms is the induction of the development of imposex in gastropods (*6*). Imposex (as an abbreviation of imposed sexual organs) is defined to be an irreversible syndrome imposing male genital tracts, such as penis and vas deferens, upon female gastropods (*7*). Gastropod imposex is known to be typically induced by very low concentrations of TBT and/or TPT (*7*–*18*). Reproductive failure is involved at severely affected stages of imposex, due to either oviduct blockage by vas deferens formation or ovarian spermatogenesis, resulting in population declines and/or mass extinction (*6*, *19*, *20*). Approximately 150 species of gastropods including the rock shell (*Thais clavigera*) have been observed to be affected by imposex in the world (*6*). Gastropod imposex is thought to be one manifestation of endocrine disruption in wildlife (*6*). Despite several hypotheses about imposex induction mechanisms, such as those involving aromatase inhibition, testosterone excretion–inhibition, functional disorder of female cerebropleural ganglia, and involvement of a neuropeptide–APGWamide (*21*–*25*), the exact physiological/biochemical pathway is still unclear.

The occurrence of reproductive abnormalities in wildlife may be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones (*26*). The nuclear receptors of intrinsic hormone systems are likely to be targets of industrial chemicals because they are originally mediators for fat-soluble, low molecular weight agents such as steroid hormones, thyroid hormones, fat-soluble vitamins, and fatty acids. Forty-eight members of the nuclear receptor family have been shown to exist in the human genome (*27*). Information on the ability of chemicals to bind nuclear receptor family members is therefore important for environmental risk assessment.

To determine if environmental pollutants can bind to members of the nuclear receptor family, we constructed assay systems for human nuclear receptors including ERα, ERβ, AR, PR, GR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, RXRα, RXRβ, RXRγ, CAR, and SXR based on a yeast two-hybrid system (*28*). In the course of the study on suspected endocrine disrupters, we found that TBT and TPT strongly enhanced the protein–protein interaction between human RXRs (hRXRs) and coactivator TIF2 to a somewhat greater extent than 9-cis retinoic acid (RA), the natural ligand of RXR.

Here, we will show the results of interaction between organotin compounds, such as TBT and TPT, and hRXR. We will also report the results of cloning of the RXR homologue from the rock shell (*T. clavigera*), its binding characteristics to both 9-cis RA and organotins, and results of the in vivo injection experiment of 9-cis RA using the rock shell. On the basis of these results, we will discuss involvement of the RXR in the development of imposex caused by organotins in gastropods.

Experimental Methods

Yeast Two-Hybrid Assay. We cloned the ligand-binding domain of nuclear receptors including ERα, ERβ, AR, PR, GR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, RXRα, RXRβ, RXRγ, CAR, and SXR by RT-PCR from human mRNA (Origin Technologies, Inc.). All sequences were confirmed to be identical to the database by sequencing. These genes were subcloned into pGBT9 (Clontech, Palo Alto, CA) so that they were in the same translational reading frame as the vector's GAL4 DNA binding domain. pGBT9-NRs and pGAD424-TIF2 were introduced into *Saccharomyces cervisiae* Y190. Transformed yeasts were incubated with test chemicals for 4 h at 30 °C, and then β-galacosidase activity was measured as described in Nishikawa et al. (*28*).

Ligand Binding Assay. The LBD of hRXRα (codons 201–693), hRXRβ (codons 275–534), hRXRγ (codons 172–455), and the rock shell RXR (sRXR) (codons 177–431) were subcloned into pGEX-4T (Pharmacia, Uppsala, Sweden). GST-RXRs fusions were expressed in *Escherichia coli* BL21 and purified according to the standard procedure (Pharmacia, Uppsala, Sweden). The purified proteins (30 μg/mL) were incubated with increasing concentrations of 9-cis-[20-methyl-^{3}H]retinoic acid (69.4 Ci/mmol, NEN Life Science Products, Inc.) with or without a 400-fold molar excess of

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TABLE 1. Body Size of Female Rock Shells (*Thais clavigera*) Used in the Injection Experiment (February 14—March 14, 2003)[a]

	control	RA	TPT
shell height (mm)	21.6 ± 1.8	21.5 ± 1.5	20.3 ± 1.4
shell width (mm)	14.5 ± 1.2	14.4 ± 1.2	14.2 ± 0.5
shell weight (g)	2.0 ± 0.5	1.9 ± 0.5	1.7 ± 0.3

[a] Mean ± standard deviation.

TABLE 2. Quality of Artificial Seawater during the Experimental Period (February 14—March 14, 2003)[a]

	control	RA	TPT
water temp (°C)	18.1 ± 0.1	18.2 ± 0.1	18.2 ± 0.1
pH	8.28 ± 0.02	8.31 ± 0.02	8.31 ± 0.04
salinity (‰)	33.5 ± 1.0	33.5 ± 0.9	33.4 ± 1.0

[a] Mean ± standard deviation.

unlabeled 9-cis RA. After incubation at 4 °C for 1 h, specific binding was determined by hydroxyapatite binding assay (*29*). Similarly, organotin compounds were used to compete for 9-cis RA in this assay to determine the binding preference for RXRs.

DNA Cloning. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using total RNA derived from male *T. clavigera*. Degenerate primers used for amplification of RXR were synthesized as follows: F-primer, 5′-TGYGARGGNTGYAARGGNTTYTTYAARMG-3′; R-primer, 5′-RAAGTGNGGVABNMKYTTVGCCCAYTC-3′. A single 390-bp fragment was obtained and sequenced. The fragment was used as a probe for screening in a cDNA library made with λ-ZAP II phagemid vector (Stratagen, Kirkland, WA). The 5′ end of the cDNA was cloned using 5′-Full RACE Core Set (Takara Bio, Shiga, Japan). The amplified products were analyzed by agarose gel electrophoresis, isolated from the gel, cloned into a pBluescript. Five independent clones were sequenced.

In Vivo Injection Experiment. The rock shell specimens were collected at Hiraiso in Ibaraki Prefecture, Japan, in December 2002 for experiments to investigate the effect of 9-cis RA. The rock shells were reared in a laboratory aquarium for approximately 2 months in artificial seawater (Senju Pharmaceutical Co. Ltd., Japan) with live mussels (*Septifer virgatus*) collected at Hiraiso as feed. Before the experiments, the rock shells were narcotized by exposure to a 72 g/L solution of magnesium chloride hexahydrate to allow the selection of females. As a male rock shell has a large penis behind the right tentacle, female shells were easily recognized by its absence (*16*, *18*). The female rock shells were divided

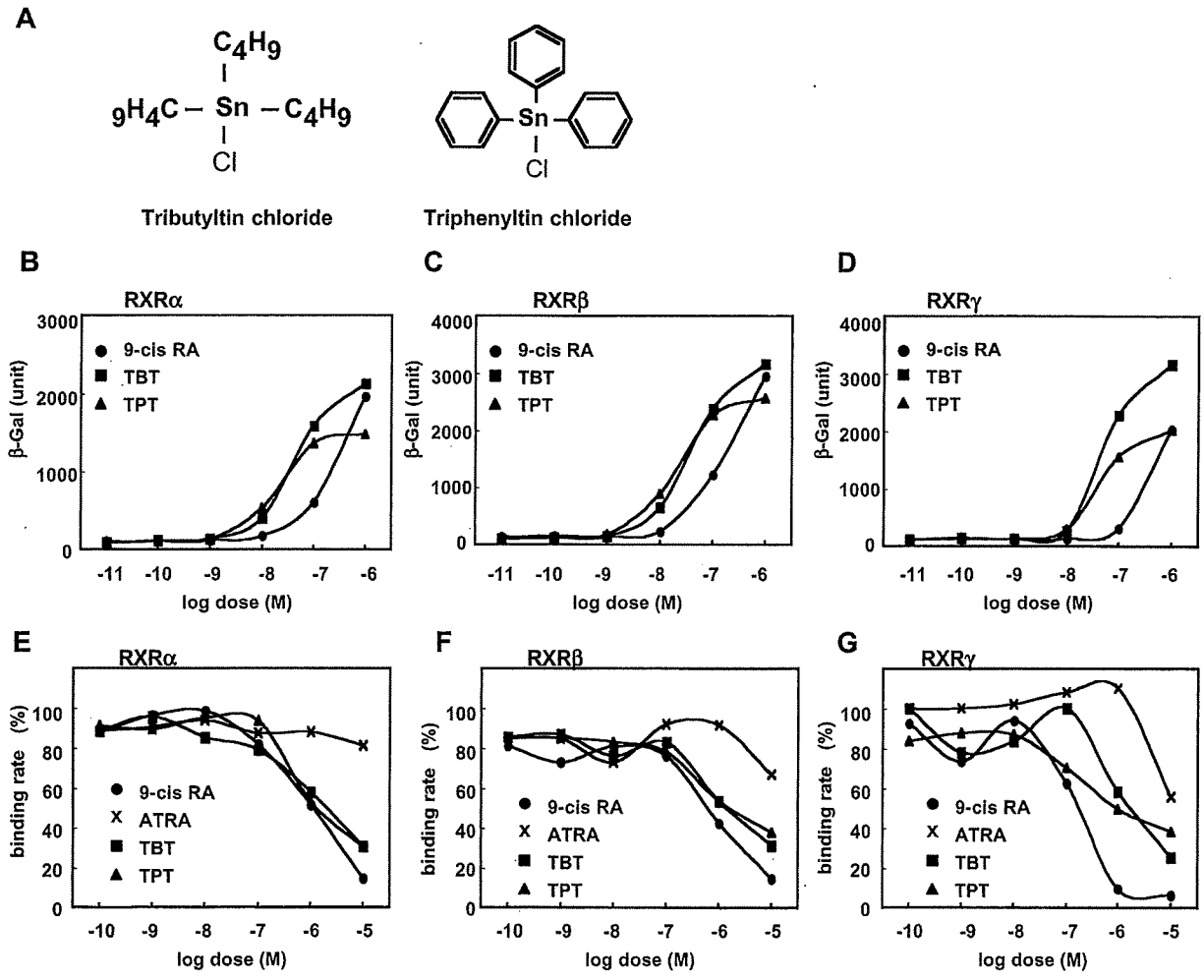

FIGURE 1. RXR activation by TBT and TPT. Structures of organotin compounds are shown in panel A. Yeast strain Y190 was transformed with GAL4AD fused to TIF2 and GAL4DBD fused to LBD of human RXRα (B), RXRβ (C), or RXRγ (D). Chemicals were added to yeast cultures in doses ranging from 10^{-11} to 10^{-6} M. Following 4 h incubation, yeasts were disrupted and assayed for β-galactosidase activity. Data points are means of three independent experiments. For in vitro binding assay, LBDs of RXRα (E), RXRβ (F), or RXRγ (G) were expressed in *E. coli* as fusion proteins with GST. Increasing amounts of chemicals were added to RXRs with 9-*cis*-[20-methyl-^{3}H]retinoic acid for competitive binding assays.

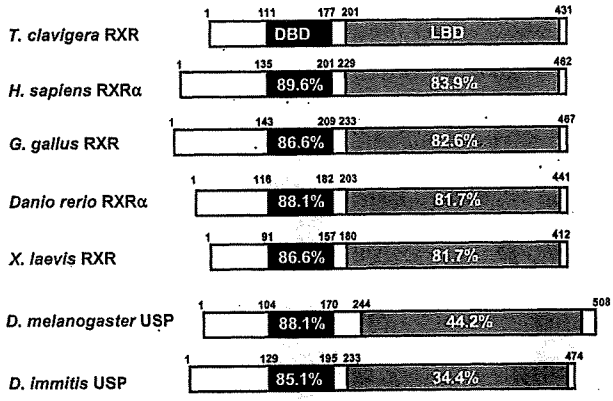

FIGURE 2. Comparison of the deduced amino acid sequences of rock shell RXR with related nuclear receptors. The similarity in the DBD and LBD between rock shell RXR and related nuclear receptors is indicated as percentage amino acid identity. The database accession numbers for the sequences are as follows: *T. clavigera*, AY704160; *H. sapiens*, NM 002957; *G. gallus*, X58997; *Danio rerio*, U29940; *X. laevis*, X87366; *D. melanogaster*, NM 057433; *D. immitis*, AF438230.

into three experimental groups of 20 animals each: for 9-cis RA injection, for triphenyltin (TPT) injection, and for control. 9-cis RA (Wako Pure Chemicals Industries, Ltd., Japan) was prepared in a fetal bovine serum (FBS; Flow Laboratories Inc.) and was injected into the foot at an application rate of approximately 1 μg/g wet wt of soft tissue of the rock shell. FBS was injected to the control animals. Triphenyltin chloride (TPTCl; Tokyo Kasei Kogyo Co., Japan, 98% pure) was used as a positive control agent and was also injected at a rate of approximately 1 μg/g wet wt of soft tissue of the rock shell. The body size of the female rock shells used in the injection experiment is shown in Table 1. After the injection of each test solution, the rock shells were kept in 2 L glass beakers in separate groups, in flow-through systems of artificial seawater saturated with oxygen (10 L/d), with live mussels as feed, for 1 month. Temperature of experimental seawater was maintained to be 18 ± 1 °C. The quality of artificial seawater during the experimental period (February 14–March 14, 2003) is summarized in Table 2. After this time, animals were removed for imposex examination (*16*). Parameters concerning gastropod imposex [the incidence of imposex (percentage occurrence of imposex individuals among females used in the experimental group), mean values of penis length (measured by automatic/digital caliper), and the vas deferens sequence (VDS) index (an index for the degree of development of vas deferens in the imposex-exhibiting female; the VDS index for the rock shell is similar to that for the dog-whelk reported by Gibbs et al.; *12*)] were calculated for each experimental group (*12*, *16*), and the statistical significance of any difference to the control group was tested. The statistical significance of the incidence of imposex was determined using Fisher's *t*-test, and an analysis of variance (ANOVA) was carried out for penis length and VDS index (*24*).

Results and Discussion

Interaction between Organotin Compounds (TBT and TPT) and hRXR. We found that TBT and TPT (Figure 1A) strongly enhanced the protein–protein interaction between hRXRs and coactivator TIF2 (Figure 1B–D) to a somewhat greater extent than 9-cis RA, the natural ligand of RXR (*30*, *31*). Because the interaction of nuclear receptor with coactivator correlated with transcriptional activity (*32*), organotin compounds, such as TBT and TPT, were thought to be agonists for hRXRs. However, TBT and TPT showed no activity to other nuclear receptors including retinoic acid receptors

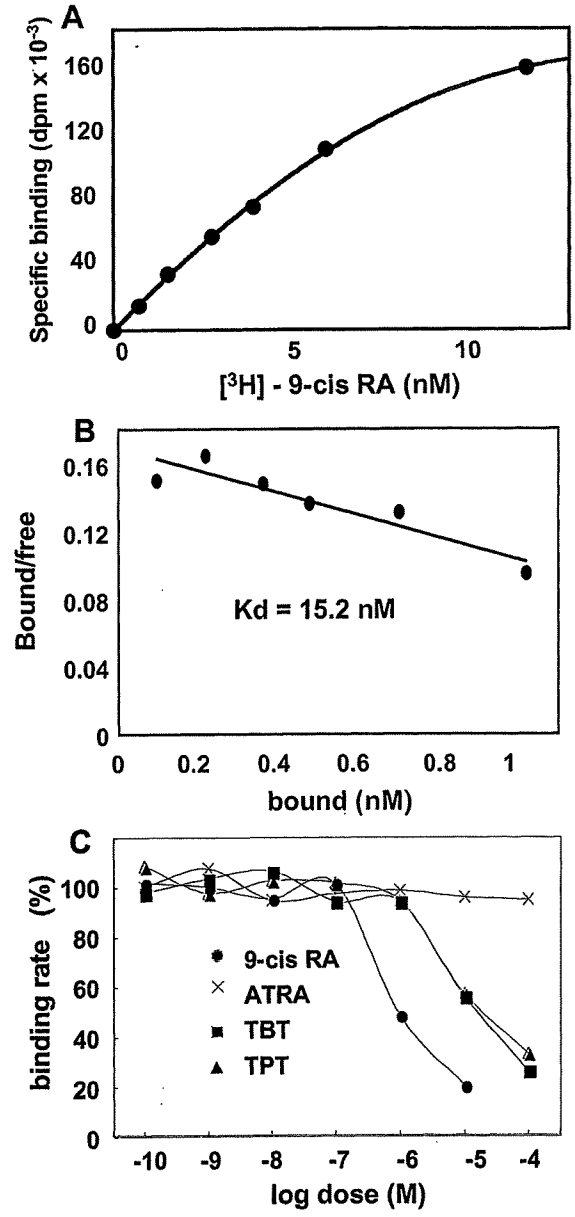

FIGURE 3. 9-cis RA and organotin compounds bind rock shell RXR in vitro. (A) The LBD of rock shell RXR expressed in *E. coli* was incubated with increasing concentrations of ^{3}H-labeled 9-cis RA in the absence (total binding) or presence of 400-fold nonlabeled 9-cis RA (nonspecific binding). Nonspecific binding was subtracted from total binding and plotted as specific binding. (B) Scatchard analysis. Specific 9-cis RA binding to rock shell RXR was transformed by Scatchard analysis and plotted. Linear regression yielded K_d = 15.2 nM. (C) Competition assay. The LBD of rock shell RXR was incubated with increasing concentrations of nonlabeled 9-cis RA, ATRA, TBT, or TPT in the presence of ^{3}H-labeled 9-cis RA.

(RARs) (*33*). While 9-cis RA is known to be a ligand for RARs as well as RXRs (*30*), organotin compounds are specific for RXRs. To confirm the binding of organotin compounds to hRXRs, we carried out an in vitro competition assay against ^{3}H-labeled 9-cis RA and found that TBT and TPT bound to RXRs as well as 9-cis RA (Figure 1E–G). The observation that TBT and TPT could act as agonists for hRXRs led us to investigate the involvement of RXR in the development of imposex in gastropods.

Cloned Rock Shell RXR (sRXR). We tried to clone the RXR cDNA from *T. clavigera*. Comparison of the RXR protein sequences in various species revealed significant similarities in the P-box in the DNA binding domain (DBD) and

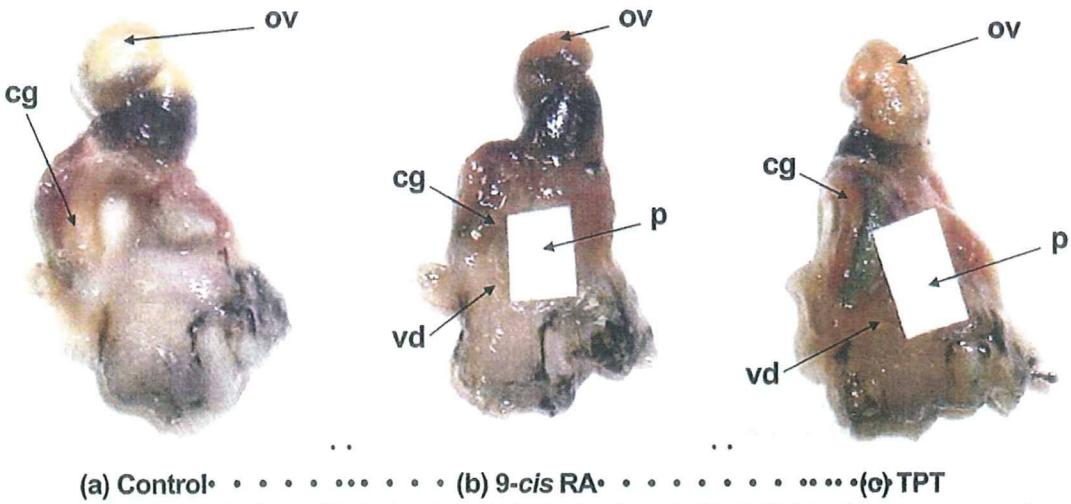

FIGURE 4. Substantial penis growth observed in the female rock shells after 1 month of 9-cis RA injections: cg, capsule gland; ov, ovary; p, penis; vd, vas deferens. (A) Neither penis nor vas deferens was observed in the control female (after shell removal). (B) Substantial penis growth as well as vas deferens development was observed in the female which received 9-cis RA injection (after shell removal; penis length: 6.06 mm). (C) Substantial penis growth as well as vas deferens development was also observed in the positive control female that received TPT injection (after shell removal; penis length: 6.50 mm). Imposex symptoms based on penis length and vas deferens sequence (VDS) index of the females that received 9-cis RA injections were clearly promoted, similar to those of females receiving TPT injections.

helix 4 in the ligand-binding domain (LBD) (*34*, *35*). By using degenerate primers deduced from these peptide sequences, we obtained a segment of *T. clavigera* RXR. Next, the *T. clavigera* cDNA library was screened to high precision using the RT-PCR product as a probe. Given that the cDNA isolated by screening was truncated, the 5′ end was amplified by RACE. Comparison of the rock shell RXR (sRXR) protein sequence with the Genebank database revealed that sRXR is closely related to vertebrate RXRs and invertebrate homologues (Figure 2). The highest homology with other species is in the DBD where 85−90% of the amino acids residues are identical (Figure 2). The LBD of sRXR also shows considerable homology with vertebrate RXRs but much less homology to ultraspiracle (USP), the RXR homologue found in *Drosophila*.

Ligand Binding Assay. Vertebrate RXRs bind to 9-cis RA, but insect USP does not (*30*, *31*, *36*). The LBD of sRXR protein, expressed after fusion with GST in bacteria, bound to 9-cis RA with $K_d = 15.2$ nM (Figure 3A,B), similar to values reported for vertebrate RXRs (*30*). These data implied that *T. clavigera* RXR could bind to 9-cis RA, even though *T. clavigera* is a gastropod mollusk. The sRXR fusion protein also bound to organotin compounds, such as TBT or TPT (Figure 3C). On the other hand, sRXR did not bind to all-trans RA (ATRA) in contrast to human RXRs that bind to ATRA even with low affinity (*30*) (Figure 3C; Figure 1E−G). The jellyfish RXR has also been reported to bind 9-cis RA with high affinity but not to ATRA (*37*).

In Vivo Injection Experiment To Examine the Involvement of RXR in the Development of Imposex in *Thais clavigera*: Effect of 9-cis RA Inducing and/or Promoting the Development of Imposex. To further verify the involvement of RXR in the development of imposex in gastropods, live female rock shells (*T. clavigera*) collected at Hiraiso in Ibaraki Prefecture, Japan (an area of low organotin contamination: see Horiguchi et al.; *18*) were injected with 9-cis RA. Results of these experiments are shown in Table 3 as well as Figure 4. Imposex was significantly induced in female *T. clavigera*, which received the injection of 9-cis RA ($p < 0.01$; Table 3), and substantial penis growth was observed in them after 1 month of 9-cis RA injections (Table 3; Figure 4). Their increased penis length and VDS index were significant when compared with controls ($p < 0.01$ and $p < 0.001$, respectively; Table 3).

TABLE 3. Incidence of Imposex (IOI), Penis Length (PL), and Vas Deferens Sequence Index (VDS) in Female Rock Shells (*Thais clavigera*) after 1 Month of Injections[a]

	control	RA	TPT
IOI (%)	10	50**	80**
PL (mm)	0.04 ± 0.13	2.87 ± 2.39**	3.77 ± 2.16***
VDS	0.20 ± 0.63	3.80 ± 0.42***	3.63 ± 0.74***

[a] Mean ± standard deviation. **, $p < 0.01$. ***, $p < 0.001$.

These results suggest that much 9-cis RA could bring about induction and/or promotion of the development of imposex in *T. clavigera* through its binding to RXR. Relatively large variance for the penis length in females that received injections of 9-cis RA may have resulted from differences in the rate of metabolism of 9-cis RA among female rock shells used in the experiment, although it is not known if *T. clavigera* inherently has a biosynthetic system for RA.

9-cis RA is the first substance, except for certain organotin compounds, that has been confirmed to induce and/or promote the development of imposex in gastopods, especially in terms of penis growth in females. As both TBT and TPT were observed to have agonistic activity to the RXR, it is strongly suggested that gastoropod imposex could be mediated by RXR.

Mode of Action of Organotins on the Development of Imposex in Gastropods. Several hypotheses have been proposed concerning the imposex induction mechanism, and they can be summarized as (i) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT (*21*); (ii) inhibition by TBT of the excretion of sulfate conjugates of androgens (*22*); (iii) disturbance by TBT of penis morphogenic/retrogressive factor released from pedal/cerebropleural ganglia (*23*); and (iv) increase in a neuropeptide, APGWamide, level caused by TBT (*24*, *25*). Experimental evidence, however, is weak for these four hypotheses. There is a lack of correlation between the time course of the increase in testosterone titers and penis growth in females in the aromatase inhibition hypothesis (*21*), and there is a possibility that the results given in support of the testosterone excretion−inhibition hypothesis (*22*) may reflect a phenomenon that is at least partly short-term and/or associated with acutely

toxic TBT concentrations (*20*). The effect of APGWamide to induce and/or promote the development of imposex also appears weak based on experimental results of incidence of imposex and penis growth (*24*, *25*).

In addition, it should be noted that substantial penis length has been observed in natural populations of imposex-exhibiting females distributed in coastal areas severely contaminated with TBT and/or TPT, as well as in females that received injections of or were exposed to TBT or TPT in the laboratory (*8*−*12*, *16*, *18*), and that little is known about basic endocrinology in invertebrates including mollusks (*38*). The penis length in female gastropods observed in the experiments given in support of the aromatase inhibition hypothesis, and the APGWamide involvement hypothesis was small (*21*, *24*, *25*). This contradiction concerning imposex development, especially penis length in imposex-exhibiting females, strongly suggested that gastropod imposex could be primarily induced and promoted by a factor other than increased androgen levels caused by aromatase inhibition or the neuropeptide, APGWamide. Moreover, there has not been any experimental evidence on purified aromatase protein itself (or aromatase at the protein level) in invertebrates, but only reports on aromatase-like activity in invertebrates including mollusks (*39*−*41*). The role of steroid sex hormones, similar to those of vertebrates, are still uncertain in invertebrates, because certain peptides have been reported to act as sex hormones in invertebrates such as *Aplysia californica* (Mollusca: Opisthobranchia), *Lymnaea stagnalis* (Mollusca: Plumonata), and *Armadillidium vulgare* (Arthropoda: Malacostraca) (*42*−*44*). In contrast, RXR is rather well-conserved from invertebrates to vertebrates (Figure 2).

In this paper, we have shown that TBT and TPT are high affinity ligands for RXR and that the natural ligand of RXR significantly caused the development of imposex in female rock shells. These results imply that RXR plays an important role in the induction/differentiation and growth of male genital tracts in female gastropods. Further studies on a heterodimer partner, coupling factors, and target genes of sRXR with molecular biological and immunohistochemical techniques are necessary to clarify the entire mode of action of TBT and/or TPT on the development of imposex in gastropods.

RXRs are key factors involved in the mediation of several hormone response systems via their association with other nuclear receptors as heterodimer partner (*45*). The knockouts of RXRs in the mouse have provided important information in the physiological functions of these receptors. RXRα null mice died in utero and exhibited a hypoplasic ventricular myocardium and ocular abnormalities (*46*, *47*). Approximately 50% of RXRβ null mice died before or at birth, and males of the remaining null mutants were sterile, owing to the aberrant lipid metabolism in Sertoli cells (*48*). On the other hand, 9-cis RA is difficult to detect in vivo, and its action is remained to be obscure (*49*). Our result that injection of 9-cis RA into female gastropods induced and/or promoted the development of imposex may provide some insight into the physiological function of 9-cis RA.

Acknowledgments

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Special Review

Percellome Projectによる毒性トランスクリプトミクスの新しい試み

Percellome Project as a New Approach to Toxicology Transcriptomics

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身の回りの物質の毒性（有害性）を予測し，その被害を未然に防ぐのが毒性学の役割である．この精度向上を目指したトキシコゲノミクス研究を実施する際に，マイクロアレイなどから細胞１個当たりのmRNAコピー数を得るPercellome法を開発した．90化合物のマウス肝初期応答データを採取し終え，新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectを展開している．

key words
トキシコゲノミクス，分子毒性学，遺伝子発現カスケード，標準化，Percellome法，３次元多層(Millefeuille）データ

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1985年東京医科歯科大学大学院医学研究科博士課程修了．人体病理学，実験病理学専攻．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌かく乱関連などの分子毒性学研究，トキシコゲノミクスプロジェクトなどを厚生労働所掌業務との有機的連携のもとに推進．

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はじめに

医薬品，食品，化粧品，生活関連用品など，身の回りの物質が我々の身体に取り込まれた際に生じる可能性のある毒性（有害性）を予測し，それらの使用に際しての被害を未然に防ぐのが毒性学の役割である[注1]（図1）．具体的には，人々の安全を確保するために使用法（用途）や使用量（残留量）を制限したり，場合によっては禁止したりするための科学的根拠を提供するが，その際，人の身代わりとして実験動物を用いる場合が多い．このような毒性学の精度向上の一環として，従来からの毒性研究（毒性症候学，毒性病理学，など）に加えてのトキシコゲノミクス（Toxicogenomics）研究が進められている．

トキシコゲノミクスでは，物質が生体に及ぼす影響をトランスクリプトームとして観測・解析する．その際，①分子毒性学を構築し種差や個体差の問題，複合暴露の問題などを解決するためには，遺伝子発現カスケードの全容解明を目指す必要がある，②形態学的に変化が現れた段階のトランスクリプトームは，遺伝子発現カスケードの最終段階に過ぎない，③形態変化の現れないごく初期段階を含む遺伝子発現カスケードを描出するためにはまとまった量のデータの蓄積が必須である，との観点から，筆者らは，マイクロアレイや定量PCRから細胞１個当たりのmRNAコピー数を得るPercellome手法と，そのデータ解析のための３次元多層（Millefeuille）システムを開発・実用化した．遺伝子発現量が共通の尺度，すなわち“コピー数／細胞”で表現されることから，検体間，実験間，マイクロアレイのバージョン間，異なったプラットホーム間，などのデータ比較が直接的に行えるようになり，数年かけて蓄積したデータの有機的活用が可能となった．現在，90種類の化学物質によるマウス肝の初期応答データを採取し終えたところである．新たな対象（反復投与，胎児毒性，吸入毒性，多臓器連携）を加えたPercellome Projectの概要を紹介する．

注1　環境への配慮も含まれる．

Ⅰ．Percellome法：細胞１個当たりのmRNA絶対量を得る方法

原理は単純である．サンプルの細胞数を計測し，外部標準mRNA（スパイクRNA）を細胞１個当たり決まった分子数だけそのサンプルに添加し，そしてRNA抽出，測定に移る．サンプルのRNAの測定値を，スパイクRNAの値を基準に，細胞1個当たりのコピー数に換算する．実際には細胞数を直接計測するのが困難なことが多いため，その代替指標として細胞核内のゲノムDNA量を用いる[1), 2)]．定量性・直線性の検証にはLBM標準サンプル（肝[L]と脳[B]を100：0，75：25，50：50，25：75および0：100に混合した5サンプルから成るセット）を用いる．なお，スパイクRNAは，5種類の枯草菌遺伝子のmRNAを濃度公比３で混合したカクテル（dose-graded spike cocktail；GSC）として用意した．高精度を要求されるDNA定量法は手作業プロトコールおよび自動ロボット（PerkinElmer JANUS）のプロトコールを準備

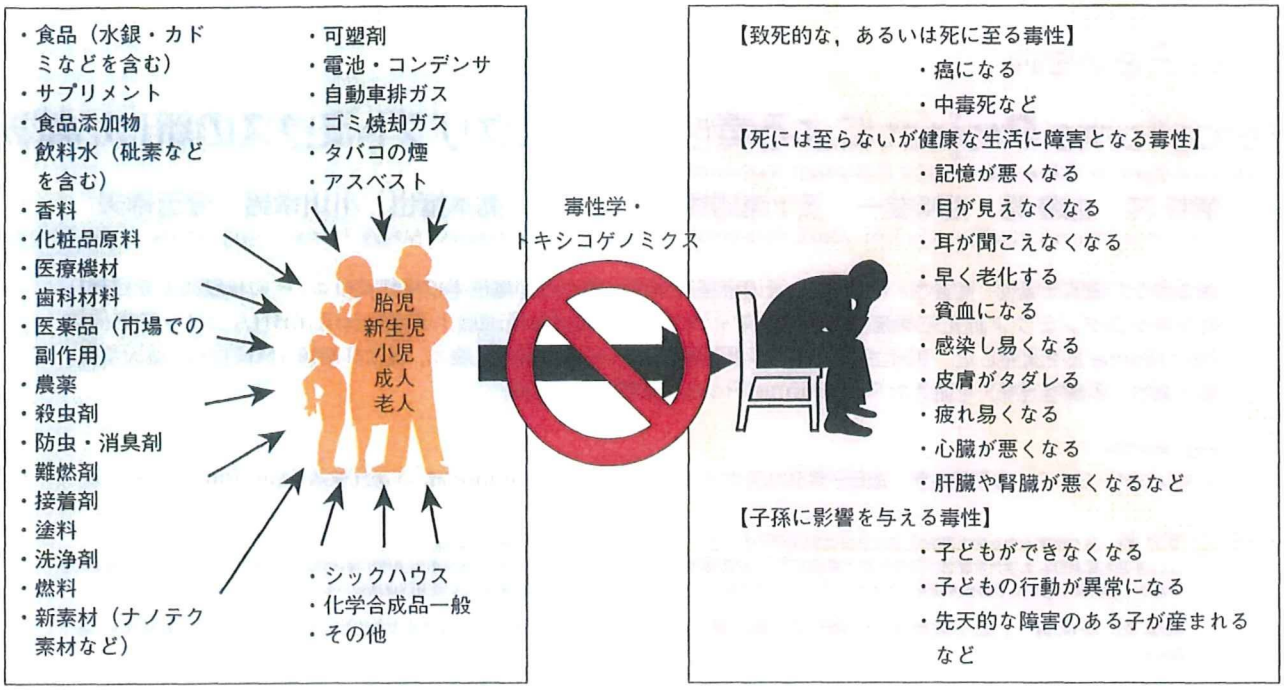

図1. 毒性学の対象

毒性学は，身の回りの物質が引き起こす障害を予測し，その発生を未然に防ぐことを目的としている．トキシコゲノミクス（毒性ゲノミクス）は，最先端の網羅的遺伝子発現解析技術を用いて，従来の毒性学の予測の精度を著しく向上，迅速化させることで，国民の健康安全の確保にさらに貢献することを目指している．

中である．カクテルとも共同研究ベースで供給可能である（連絡先：kanno@nihs.go.jp）．また，ERCC（The External RNA Control Consortium）と連絡をとるとともに，国際的標準化への関与を深めるため平成18年度厚労科研費「医薬品などの有効性・安全性評価に資する遺伝子発現解析の国際的標準化に関わる研究（H18-特別-指定-023）」を立ち上げた．現在，この他にシックハウス症候群を考慮した低用量域での吸入毒性トキシコゲノミクス，1匹のマウスから多臓器を採取しそれらの連携状況をトランスクリプトームから解析する多臓器トキシコゲノミクスを開始し，特徴的な遺伝子について組織内の発現分布を*in situ*ハイブリダイゼーションで確認する作業を並行している．また，下記の3次元データをweb公開するサーバを整備し，一部の化合物から3次元多層（Millefeuille）データを順次閲覧可能とした（http://toxicomics. nihs.go.jp/db/）．

Ⅱ．3次元多層（Millefeuille）データシステム：生物系研究者に優しいデータ可視化と解析

医薬品を含む毒性既知の90化合物について単回経口投与後のトランスクリプトームデータを取得して，初期応答遺伝子カスケードを解析するための基盤データベースを構築した．現在，第二段階として反復暴露データ集積を開始した．データは，用量軸，時間軸，および遺伝子発現軸から成る3次元表示により，遺伝子発現の用量および時間に依存した変化を1枚の曲面として表すことで可視的に変化を判別しやすいように配慮した（図2）．これにより，コンピュータが選び出した遺伝子クラスターの中身を確認する際，特に，mRNAの合成分解のスピードなどの知見から生物学的にありえないパターン（用量軸の方向にも時間軸の方向にもジグザグな変化など）を排除する際に威力を発揮している．

1つの実験から排出されるGeneChip約50枚のデータを一括処理する能力を持ったPercellome自動換算・データ品質管理（QC）に関わるソフトウエアに加えて，3次元多層（Millefeuille）データに最適化した，発現パターン類似性による候補遺伝子検索，およびそれを発展させた教師無しクラスタリング[3]を中心とした解析システム（MF System，MFシリーズ，開発：相﨑 健一）を独自に実用化し，開発継続中である（図3）．これらにより，データQCはその日のうちに，基本的な発現情報検索から全遺伝子の教師無しクラスタリングまでを3日間で完遂できるものとなっている．

この基本解析を用いて，発現パターンによって分類された候補遺伝子リストが多数生成される．一部の幸運な例ではただちに新規と思われる毒性関連反応を見いだすことができた．またそうでない場合のための1つの補強手段とし

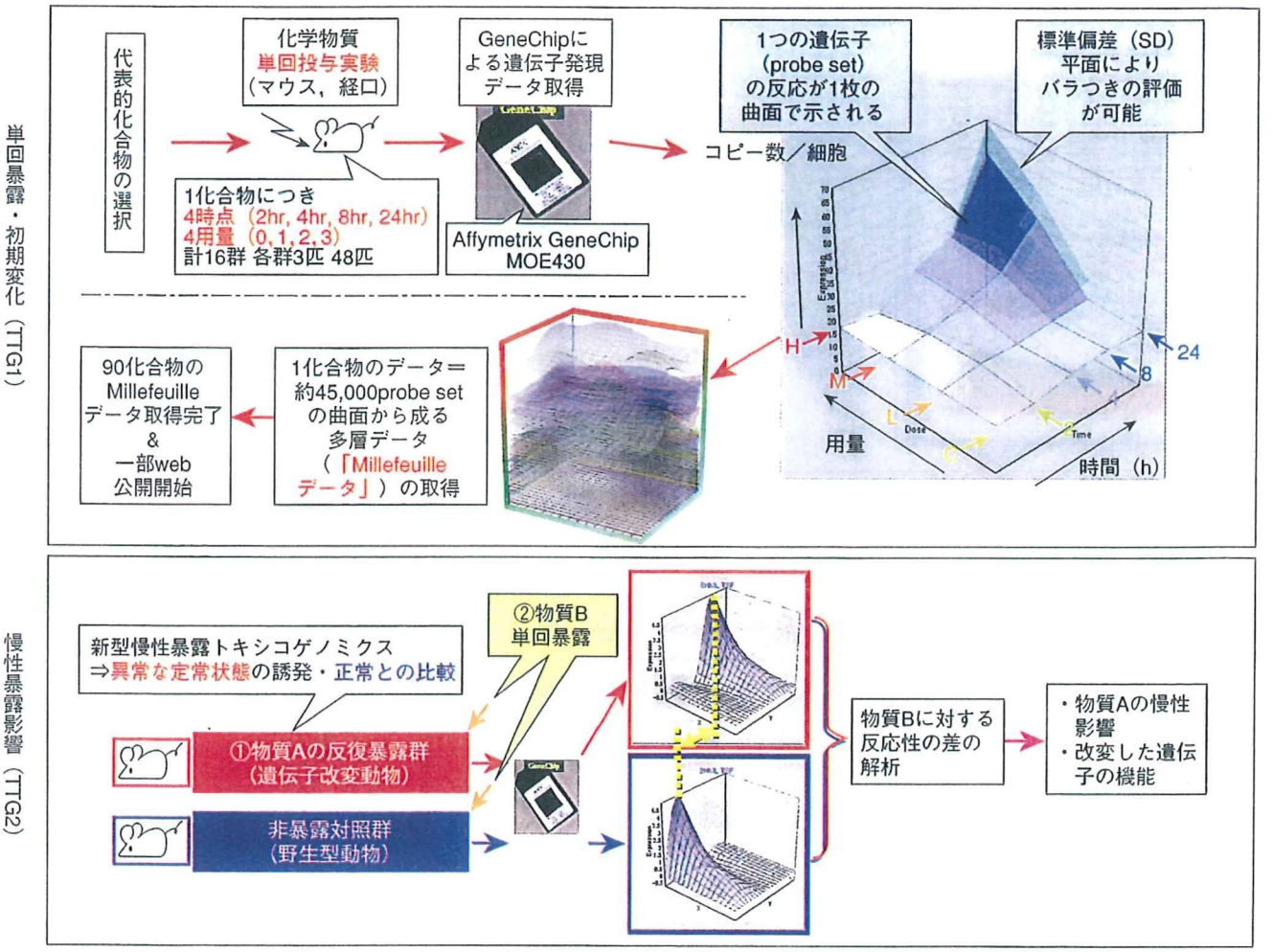

図2. Percellome法と3次元表示による多層（Millefeuille）データシステムを用いたプロジェクトの根幹部分の概要
単回投与による遺伝子発現初期変化データを90化合物について取得（上段）．現在，反復投与の影響を検討中（下段）．H；高用量（high），M；中用量（medium），L；低用量（low），C；コントロール（control）．

て，Gene Ontologyなどの既存知識を利用して候補遺伝子リストの理解を支援するソフトウエア（MF GoPlot）を用意した．このツールは一種の化合物クラスタリングとしても利用することができる．

さらに候補遺伝子リストを基に複数化合物間比較を行い，複数条件下においても同期して発現する遺伝子群を自動抽出するシステムも開発済みである．本システムで得られた同期遺伝子群はシグナルカスケードの構成単位である可能性があり，データベース化しつつ，その解析を進めている（5TB規模のデータベース部分および，大量計算アルゴリズム実装は（株）NTTコムウェアおよび（株）日本NCR/Teradataとの共同開発による）．

Ⅲ．Percellome手法のリアルタイムPCRを含む他のプラットホームへの適用

Percellome手法は，GSCの受け入れ条件を整えることにより，様々なプラットホームに適用可能である．その1つとして最も定量性が高いとされるリアルタイムPCR（ABI PRISM 7900 HT・96ウェルプレート）への適用例を示す．現行のRT-PCR絶対定量法では，遺伝子ごとに検量線が必要であり，多数のサンプルについて多数の遺伝子を検討するには不向きである．Percellome RT-PCRでは，マイクロアレイと同様の原理を用いる．すなわち，サンプル破砕液に，その細胞数に比例する量のスパイクカクテル（GSC）を添加し，それらのCt値をPCRプレートごとの検量線とすることにより，測定したい遺伝子のCt値を細胞1個当たりのmRNAコピー数に換算する．これにより，GAPDHやActinなどのハウスキーピング遺伝子が変動してしまう際の問題，例えば，少数の遺伝子を検討する際にGlobal normalization法を適用し難い問題などが解決される．共通サンプルを測定しデータを比較することにより，Affymetrix GeneChipのPercellome結果と9割程度の整合性が確認され，

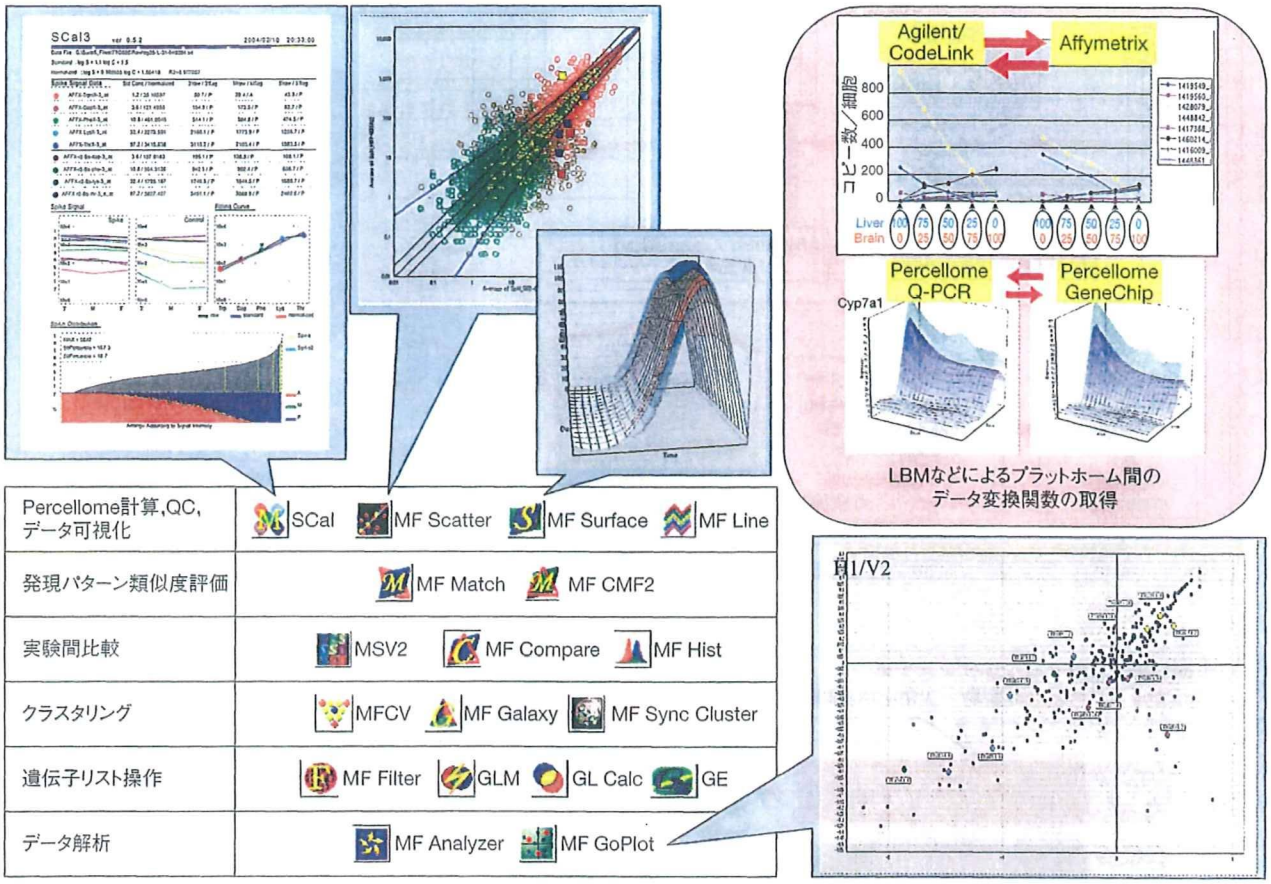

図3．3次元多層（Millefeuille）データの解析などに用いる独自開発プログラム群

品質管理とともにPrecellome計算を自動的に実施するSCal，Plotソフトウエア，3次元曲面の描画ソフト（MF Surface），など．右上はプラットホーム間のデータ変換情報の得方を示す．LBMを用いる方法（上段）と，実際の実験サンプルを用いる方法（下段）がある．いずれも，一度，両方のプラットホームでそれらのサンプルを測定する必要がある．

GeneChipとPercellome RT-PCRとの間でのコピー数の換算式がいくつかの遺伝子について得られている．この他に，Agilent社製の単色マイクロアレイとCodeLinkアレイにGSCを測定可能なカスタムアレイを用意し終え，LBMサンプルのデータなどをもとに，これらとの間の換算式も得つつある（図3右上）．

Percellome法は，Affymetrixの新しいエキソンアレイの定量性・直線性の検討にも適応可能である．Affymetrix社のHuman Exon 1.0 ST Arrayと従来型の発現アレイHuman Genome U133 plus 2について，性質の異なるヒト癌細胞株2株から調製したLBM様標準サンプル（100：0，75：25，50：50，25：75および0：100混合5サンプル）による比較を行い，両アレイ間の相関性の高いprobe setを多数検出することができた．また，既知のエキソンに対して設計されたprobe setでは発現が見られ，イントロンに対して設計されたprobe setでは発現が見られない，あるいは，既知のsplicing variantに対応したprobe setの発現が検出された，などの基本性能が確認された．しかし，Percellome法を適用して未知のsplicing variantの検出力を向上させるためには，現状では各エキソン間の定量性に問題があることが示唆された．定量値を算出する補正アルゴリズムの開発など，何らかの対策が必要であることが考えられ，現在，Affymetrix社に確認を行っている．

Ⅳ．核内受容体原性毒性のPercellomeトキシコゲノミクス解析

受容体原性毒性とは，化学物質が受容体（リガンド依存的転写因子を含む）に選択的に結合してシグナルをかく乱し，その結果生じる有害性を指す．代表例としてはダイオキシンが挙げられる．AhR（Arylhydrocarbon receptor）ノックアウトマウスでは，ダイオキシンを大量に投与しても毒性がほとんど観察されない．すなわち，野生型マウスがダイオキシンで死ぬメカニズムには，AhRが必須であり，AhRからの異常なシグナルがマウスを死に至らせていることに

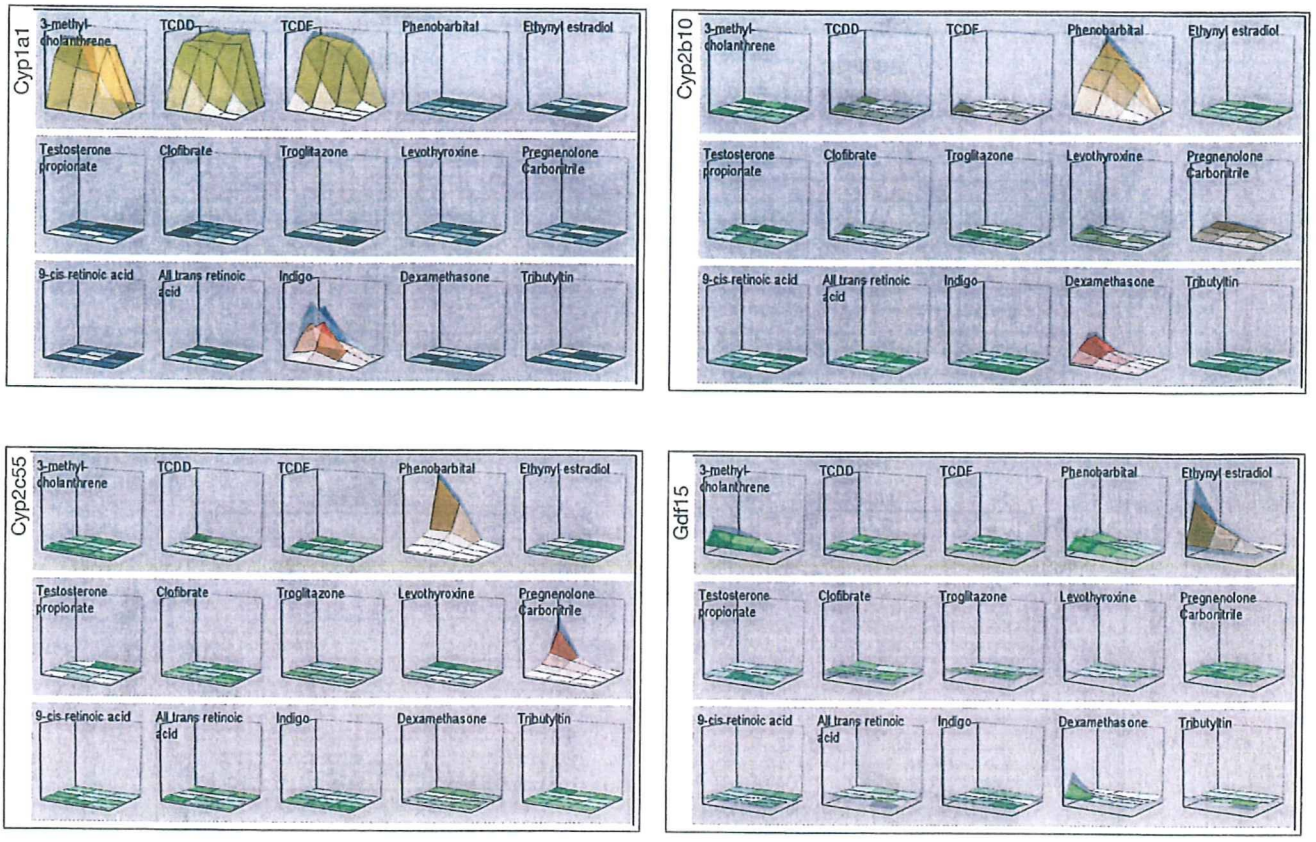

図4. 化合物間の発現比較

15種類の核内受容体リガンド化合物（各3次元グラフ内に表示）によるCyp1a1（左上），Cyp2c55（左下），Cyp2b10（右上）および，Gdf15（右下）の遺伝子発現を3次元表示したもの．各軸は，図2のとおり．縦軸のスケールは遺伝子ごとに共通．リガンドに選択的な遺伝子の発現が確認される．

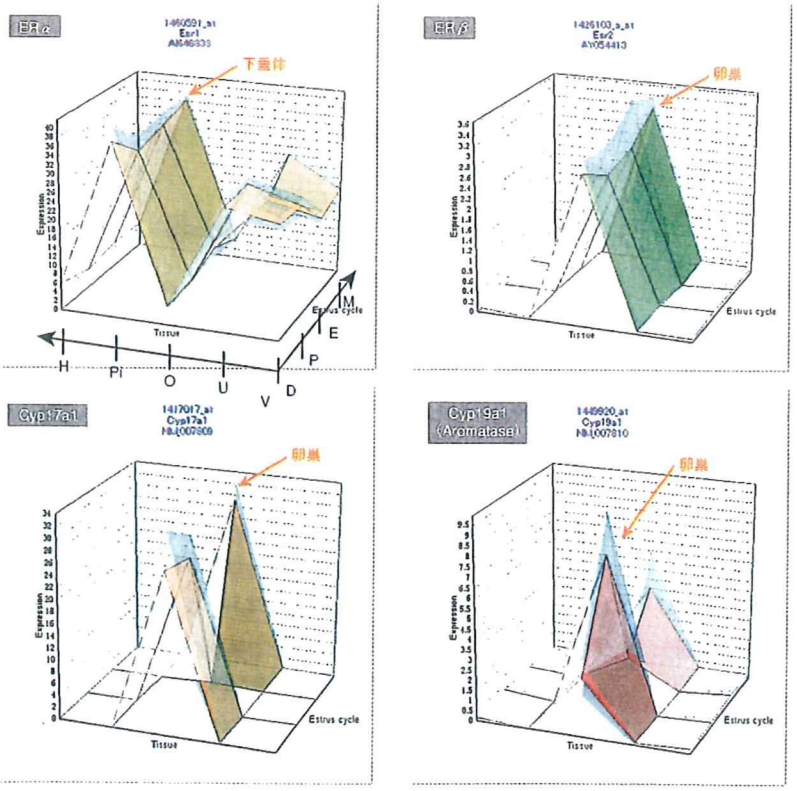

図5. 臓器間の発現比較

マウスの性周期（Diestrus，Proestrus，Estrus，Metestrusの4日間で1周期）ごとの視床下部（H），下垂体（Pi），卵巣（O），子宮（U）および膣（V）における，ERα，ERβ，Cyp17a1（steroid-17α-hydroxylase），およびCyp19a1（Aromatase）の遺伝子発現変動を3次元表示したもの．後二者の酵素は卵巣において周期性を持って発現している．

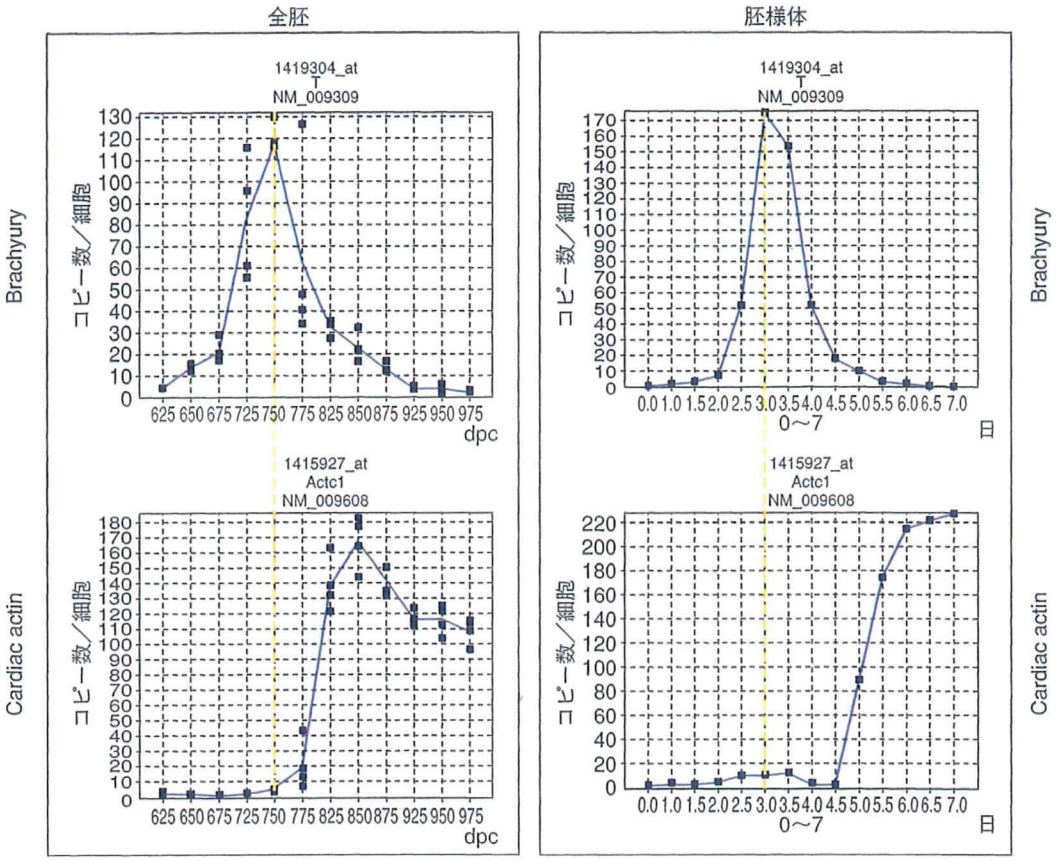

図6. マウス胎児（全胚）と胚様体の発現比較

マウス全胚の胎生6.25日～9.75日までの遺伝子発現と，胚様体の1日～7日目までの遺伝子変動の網羅的データベースから，初期中胚葉分化マーカーであるBrachyury遺伝子と，Cardiac actin遺伝子の経時変化を示す．

なる．エストロジェン活性化学物質による有害影響（内分泌かく乱化学物質問題）も同様にER（estrogen receptor）を介する受容体原性毒性と考えられ，胎生期にERを発現する組織が，低用量シグナルかく乱影響の重要標的であると考えられている．

ここでは，受容体原性毒性研究の基盤として，Percellome手法を適用して，①核内受容体作動性物質によるマウス雄肝臓の遺伝子発現変動，②性周期に伴うマウス雌生殖器遺伝子発現変動，③生後の発達過程におけるマウス雌生殖器遺伝子発現変動，の3種類のデータベースを構築した．例えば，①では10種類の核内受容体に作用する典型物質について，単回経口投与後，2，4，8，24時間目の変動を解析し，Ethynyl-estradiolがGDF15，TCDDがCyp1a1，9-cis Retinoic AcidがCyp26a1，DexamethasoneがCyp2b10，ClofibrateがCyp4a14，PCNがCyp2c55など，各々の受容体に特徴的な遺伝子発現を誘導するところがとらえられている（図4）．②の性周期データベースは視床下部，下垂体，卵巣，子宮，膣を対象としており，性周期との関連が網羅的にとらえられている（図5）．これらのデータベースは，今後，各種の候補物質が引き起こす変化を詳細に解析する際の基準として利用される．

V．発生トキシコゲノミクスへの応用

発生毒性学は，個体発生過程におけるダイナミックな遺伝子発現調節の分子機構を把握することにより，さらに正確なものに補強されると考える．現在，C57BL/6マウス胚の器官形成期初期にあたる胎生6.5～9.5日（プラグ確認日：0.5日）の，①全胚の遺伝子発現変動解析，②遺伝子欠失マウス全胚との比較，および③標的が明らかな既知発生毒性物質投与による本データベースの具体的な適用，を実施している．①についてはすでに0.25日間隔（Time point 計12点）の遺伝子発現データベースを得て，②遺伝子欠失胚のデータといくつかの注目すべき遺伝子についてはwhole mount ISHを用いた発現の検証を加えた．これと並行して，ES細胞からhanging drop法で得た胚様体の0.5日間隔の遺伝子発現データとの比較を実施している．個体発生に関与

する遺伝子群の多くは経時的に激しく変化しており，既知発生毒性物質投与実験については標的遺伝子シグナルカスケードを解析中である（図6）．

おわりに

ノーザンブロットでは実験サンプルにだけバンドが見られ，対照サンプルには遺伝子発現がないという結果を得ても，細胞1個当たりで定量してみると，対照が10コピーに対して実験サンプルが20コピーである場合がある．“無”が“有”になったのではなく，“10”が“20”になったのである．

さて，筆者らの属する毒性学でも，医学の分野でも，疾患概念や毒性概念が整理され，患者や実験動物を診断する際には，まず，そのどれに当てはまるかを検討する．すなわち，どの“典型”に近い症例であるかを検討することから始まることが多い．

しかし，最近の医学・生物学には多因子疾患・多因子形質発現制御の概念が導入され，今から何年かの後には，“21世紀初頭までは，患者の遺伝子多型を調べずして治療を行っていた時代”として，“血液型を調べずに輸血していた時代”と並び称されるようになる可能性がある．このような多因子概念が定着すると，その多くは，“有（100%）”“無（0%）”の組み合わせではなく，“70%”“50%”“90%”といった半端な数の組み合わせであることが考えられる．すなわち，今までの離散値的な“典型”例を基準とするアプローチから，連続値的な病態“スペクトラム”を直接扱うアプローチに変革していく可能性が考えられる．その際の網羅的データの解析とその蓄積の必要性を考えるとき，遺伝子発現データの定量化・標準化という問題は，今まで以上に重みを増すと考えられる．生命現象の網羅的解析にはトランスクリプトームだけでは不十分であることは自明であるが，この定量性を確保することは，これから実現されるであろう網羅的プロテオミクスなどの基盤としても重要ではないかと考える．

マイクロアレイなどから得られるトランスクリプトーム情報が，今後の医薬品審査や化学物質の安全性評価の際に必須なものとなる時代がすぐそこまで来ていることを念頭に，筆者らはPercellome法をさらに展開し，Percellome Projectデータベースを可能な限り高精度に保ちつつ毒性学的な内容を充実させるべく最大限の活動を継続して行く所存であるが，この技術，あるいは研究内容が毒性学以外の研究分野にもお役に立つことができれば幸甚である．

謝辞 本システムの開発とプロジェクトの遂行に当たっては，当毒性部の全メンバー，特に松田菜恵，辻 昌貴，森田紘一，今井あや子，安東朋子，安部麻紀，森山紀子，近藤優子，青柳千百合，相原妃佐子，渡辺 忍の各氏の卓越した働きに深謝する．本研究は厚生労働科学研究費補助金H13-生活-012，H13-生活-013，H14-トキシコ-001，H15-化学-002，H18-化学・一般-001などによる．

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Original

Observation of Preputial Separation is a Useful Tool for Evaluating Endocrine Active Chemicals

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Abstract: Flutamide, *p,p'*-dichlorodiphenyldichloroethylene, vinclozolin, diethylstilbestrol, ethynylestradiol and tamoxifen were administered by gavage to pregnant Sprague-Dawley rats on gestational days 14–17 or 18–21, and to male offspring on postnatal days 1–5, 17–21 or 35–39. The influence on the sexual maturation was assessed by preputial separation. Cleft phallus with hypospadias was induced by prenatal exposure to 10 mg/kg flutamide on gestational days 14–17 and 18–21, or administration of 100 mg/kg vinclozolin on gestational days 14–17 to the dams. The day of preputial separation in these offspring could not be determined, because complete separation did not occur. Prenatal exposure of males to other chemicals did not affect the preputial separation. Postnatal exposure of 10 and 30 mg/kg flutamide and 30 mg/kg vinclozolin led to delays of preputial separation. A marked delay was observed in males exposed to 100 μg/kg of ethynylestradiol or 3 mg/kg of tamoxifen on postnatal days 1–5. Diethylstilbestrol, 300 μg/kg, administration on postnatal days 1–5 and 35–39 caused a delay in preputial separation. These results indicate that observing preputial separation is useful for evaluating anti-androgen treatment in the prepubertal period, and estrogen-related chemical treatment from the neonatal period. (J Toxicol Pathol 2005; **18**: 141–157)

Key words: preputial separation, rat, flutamide, vinclozolin, ethynylestradiol, diethylstilbestrol

Introduction

Preputial separation, which is the separation of the prepuce from the glans penis, is used as an indication of puberty in the male rat. Histological observations on the progress of preputial separation after cornification at the lining of the prepuce and surface of the glans penis were well described using Long-Evans rats in 1942[1]. We showed similar histological changes in Sprague-Dawley rats examined from postnatal day (PND) 6 to PND 56[2]. Preputial separation is thought to be dependent on androgens, since castration blocks preputial separation and the addition of testosterone (TS) or dihydrotestosterone (DHT) nullifies the effect of castration[1,3]. In recent years, various *in vivo* screening assays have been developed for detecting endocrine disrupting chemicals. The uterotrophic assay is a method for detecting estrogenic or anti-estrogenic effects of chemicals on the weight of the uterus using immature or ovariectomized female rats. The Hershberger assay is a screening test to detect androgenic or anti-androgenic effects of chemicals on the weight of castrated male reproductive organs such as the ventral prostate and seminal vesicles. The enhanced OECD Test Guideline 407 is a draft, new version of the Repeated Dose 28-day Oral Toxicity Study in Rodents, and is designed to detect the endocrine effect of chemicals. Also, the rodent 20-day thyroid/pubertal male assay has been proposed for evaluating chemicals influencing male puberty. In this assay, weaning male rats are continuously dosed by gavage beginning one week before puberty (which occurs at about PND 40) until PND 53, and their puberty is measured by determining their age at preputial separation. Both estrogenic and anti-androgenic chemicals may induce delays in male puberty. Although the observation of preputial separation is a useful tool for detecting sexual maturation, the adequate administration period or relationship between dose and effect has not been sufficiently investigated. Accordingly, we performed a preliminary study to find out the suitable administration period or the most sensitive period using the following well-known chemicals: flutamide, *p,p'*-dichlorodiphenyldichloroethylene, vinclozolin, diethylstilbestrol, ethynylestradiol and tamoxifen. Flutamide (FLU)[4,5] is an anti-androgenic drug and is used in the Hershberger assay as the positive control agent. *p,p'*-dichlorodiphenyldichloroethylene (DDE)[4–7] and vinclozolin (VZ)[7,8] are anti-androgenic chemicals and are used in the OECD validation study of the Hershberger assay to verify

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the effectiveness of this screening assay. Diethylstilbestrol (DES) is an estrogenic compound and has been used in various experimental studies. Ethynylestradiol (EE) is a synthetic estrogen and is used as a positive control in the uterotrophic assay, and tamoxifen (TAM) is used as a positive control for the anti-estrogenic effect in this screening assay.

To determine the administration period, the following reports were used as references. Induction of hypospadias has been reportedly caused by anti-androgens[9]. Puberty is undetermined in males with hypospadias because complete separation in the glans penis is not evident in these animals. A sensitive prenatal period for hypospadias is known to exist, thus, we selected gestational days (GD) 18–21 as the low sensitive period for prenatal exposure. We selected GD 14–17 as the high sensitive period for comparative study, but the high dose group of some chemicals may not be used for the observation of preputial separation. To examine the effects on the neonatal period, newborn rats were orally administered test chemicals on PND 1–5. Leydig cells of neonatal rats are known as fetal Leydig cells, and adult type Leydig cells appear from about PND 14[10]. It was reported that male rat serum testosterone levels reach a maximum on GD 19, decrease on PND 12, and then increase[11]. Thus, we also chose to administer the test chemicals during PND 17–21 and PND 35–39 (prepubertal period). The 4 or 5 days of administration period in the present study is short compared to the Hershberger assay (10 days) or pubertal male assay (20 days), thus the effect on the animals is thought to be less than that seen in these assays. The purpose of this preliminary study was to find out the sensitive period for the screening assay, and more prolonged administration may show more pronounced effects on the animals. The number of animals in each group was determined referring to the other screening assays such as the uterotrophic assay (6 female rats in a group), the Hershberger assay (6 male rats in a group), and the Enhanced OECD Test Guideline 407 or pubertal male assay (10 male rats and 10 female rats). The number of pups and litters may be insufficient to determine the effects of unknown chemicals, but the purpose of the present study was to obtain the suitable period of administration for a screening study by the observation of preputial separation, thus we performed the preliminary study using the experimental design described in the following section.

Materials and Methods

Animals and housing conditions

Sprague-Dawley rats (Crj:CD (SD) IGS), 210 males and 421 females, 11 weeks of age, were obtained from Charles River Japan, Inc. (Atsugi, Japan). All animals were acclimatized to laboratory conditions and quarantined for about one week before mating. Rats used for this study were selected based upon their general appearance and behavior during the acclimatization period. Animals were housed individually in wire-bottom metal cages (220 × 270 × 190 mm) and kept in a barrier sustained animal room that was maintained at 21.0 – 25.0°C and 40.0 – 75.0% relative humidity with a 12-hour artificial light cycle (lighting from 7:00 to 19:00). The room air was changed fifteen times per hour, and a commercial diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan), and water (Hadano City) were available *ad libitum* throughout the study. The Animal Use Committee of the Hatano Research Institute approved the study protocol.

To obtain pregnant animals, 12-week-old females were cohabited overnight on a 1:1 basis with males 12 weeks of age or older. Females were considered to be at GD 0 when daily examination revealed a vaginal plug. All pregnant animals were housed in cages with animal bedding, PAPER CLEAN® (Japan SLC, Inc., Shizuoka, Japan), from GD 18 and allowed to give birth. The dams and pups were housed in wire-bottom metal cages after postpartum day 10.

For prenatal exposure, pregnant females were randomly assigned to groups consisting of 3 to 5 based on body weight before administration. For PND 1–5 exposure (PND 0 is the day of delivery), all female pups were discarded on PND 1 and the number of males per litter was adjusted to 5. The litters were then allocated to groups consisting of 3 or 4 litters (except for one group consisting 2 litters) based on pup mean body weight. On PND 6, the number of pups for prenatal or neonatal exposure and that for premature exposure was adjusted to 4 males per litter. For PND 17–21 and 35–39 exposure, litters were allocated to groups a few days before administration based on mean body weight. The numbers of litters or pups were decided referring to the other screening assays.

Chemicals and treatment

FLU, DES, EE and TAM were purchased from Sigma-Aldrich (St. Louis, MO), DDE was obtained from Aldrich Chemical (Milwaukee, WI), and VZ from Wako Pure Chemical (Osaka, Japan). Each chemical was dissolved in corn oil (Nacalai Tesque, Inc., Kyoto, Japan). Dosage levels for FLU were 1, 10 and 30 mg/kg/day, for DDE 10, 30, 100 and 300 mg/kg/day, for VZ 10, 30 and 100 mg/kg/day, for DES 0.1, 1, 10, 100 and 300 μg/kg/day, for EE 10 and 100 μg/kg/day, and for TAM 0.03 and 0.1 mg/kg/day (prenatal exposure) or 0.3, 1 and 3 mg/kg/day (postnatal exposure). The dosages employed in this study were based on the results from preliminary studies or those reported in the literature.

The chemicals were orally administered by gavage (5 or 10 mL/kg BW) to pregnant female rats on GD 14–17 or 18–21, or to males on PND 1–5, 17–21 or 35–39 (Fig. 1). In addition, EE was administered on PND 6–10 or 11–15. The control animals were administered vehicle corn oil orally, at the same time periods and volume as the test group. An ATOM indwelling feeding tube (Atom Medical, Tokyo, Japan) was used for neonatal administration as described by Watanabe[12] and a stomach tube was used for adult and premature animals.

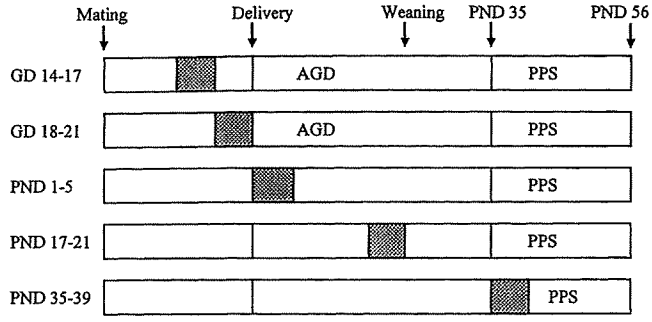

Fig. 1. Schedule of prenatal and postnatal treatment.
▒: period of chemical exposure, GD: gestational day, PND: postnatal day, AGD: measurement of anogenital distance on PND 6, PPS: observation of preputial separation from PND 35.

Examination and measurement

Body weights of pregnant females were measured on GD 0, on the day of grouping before administration, and during the administration period. Body weights of males were measured on PND 0, 6, 22, 35, 56, the day of complete preputial separation, on the day of grouping, and during the administration period. Body weights on PND 0 to 6 were measured as mean value of each litter, and after PND 22 they were measured individually. The anogenital distance (AGD) of prenatally exposed male pups was measured on PND 6 before adjusting to 4 males per litter, with a digital micrometer (reproducible precision of 0.01 mm, Digimatic caliper CD-15CP, Mitutoyo Corporation, Kanagawa, Japan). The progress of preputial separation in the males was observed macroscopically[13] from PND 35. Surviving males were sacrificed under anesthesia on PND 56 and autopsy was performed. Male fatalities were autopsied as early as possible after they were discovered. Following the macroscopic examination, the testes, epididymides, ventral prostate, seminal vesicles, prepuce and penis were excised and fixed in 0.1 mol/L phosphate buffered 10% formalin solution. Weights of the testes, epididymides, ventral prostate, and seminal vesicles of sacrificed animals were measured on the next day. Representative organs of 169 cases were embedded in paraffin, and the sections were then stained with hematoxylin-eosin (H&E) for histopathological examination. To observe early stage hypospadias some of the discarded males were sacrificed on PND 6 and examined histologically as described above.

Statistical analysis

Body weights on the day of preputial separation and autopsy, the day of preputial separation, organ weights, AGD, and correction values of AGD [AGD (mm)/ $\sqrt[3]{\text{body weight (g)}}$ of pups] were statistically analyzed using the litter as the unit. Body weights on the day of preputial separation, the day of preputial separation, and organ weights in postnatally administered males were also analyzed using the individual values. These data were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect significant differences among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, the Kruskal-Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. The day of preputial separation and the body weights on the day of preputial separation between the two groups were analyzed by Student's or Welch's t-test. Comparisons between groups were made using $P \leq 0.05$ as the level of significance. When preputial separation was not complete on the day of autopsy, the day of separation was set as day 56 for the analysis. The correlation between the day of preputial separation and body weight was analyzed using Pearson's correlation coefficient.

Results

Prenatal exposure

1) Effects on the pregnant females

In the groups exposed to 300 mg/kg of DDE on GD 14–17 or GD 18–21, body weights decreased during the administration period and either the pregnant females or their pups died. Depression of the weight gain was also observed in the 100 mg/kg DDE group exposed on GD 18–21, and all the pups in 3 litters out of 4 died. Deaths of pregnant females or pups were observed in the VZ, DES and TAM groups. Although depression of weight gain was observed in the EE group, all females survived and delivered pups. In the VZ, DES and TAM groups, one or two females delivered before the last day of administration period, and these females were excluded from the experiment.

2) Malformations and inflammatory lesions of the genital organ

The incidence of malformations and inflammatory lesions after prenatal exposure to chemicals are summarized in Table 1. On macroscopic examination, the glans penis of control males was covered with prepuce, and the prepuce could be completely retracted to expose the glans penis before PND 56 (Figs. 2A, 2B). The prepuce of males from dams exposed to 10 mg/kg of FLU on GD 14–17 had a cleft at the ventral aspect (cleft prepuce) and the glans penis was observed from the cleft. The ventral part of the penis was incompletely formed (cleft phallus) and the os penis was often exposed. The external urethral orifice of males with a cleft phallus opened at the ventral surface of the penis (hypospadias). Hypospadias was usually observed with cleft phallus. The incidence of a cleft prepuce was 25% (3 cases in 2 litters) and the incidence of a cleft phallus was 58% (7 cases in all of the 3 litters), although a cleft prepuce was usually observed with a cleft phallus. Five males had no cleft on their prepuce or phallus. Although there was no cleft at the prepuce of males from dams exposed to FLU on GD 18–21, the ventral part of the penis was incompletely formed (cleft phallus); the incidence of the cleft phallus in this group was 25% (3 cases in one litter).

Table 1. Malformation and Inflammatory Lesion in Genital Organ of Male Rats Prenatally Exposed to Chemicals

Chemical	Dosing period	Group	Males	Litters	Cleft prepuce	Cleft phallus	Ectopic testis	Hypoplasia of prostate	Prostatitis/ Vesiculitis
FLU	GD 14-17	Control	12	3	0	0	0	0	0
		1 mg/kg	12	3	0	0	0	0	0
		10 mg/kg	12	3	3 (2)	7 (3)	2 (2)	0	0
	GD 18-21	Control	12	3	0	0	0	0	0
		1 mg/kg	12	3	0	0	0	0	0
		10 mg/kg	12	3	0	3 (1)	0	0	7 (3)
DDE	GD 14-17	Control	12	3	0	0	0	0	0
		10 mg/kg	12	3	0	0	0	0	0
		30 mg/kg	16	4	0	0	0	0	0
		100 mg/kg	16	4	0	0	0	0	0
	GD 18-21	Control	14	4	0	0	0	0	0
		10 mg/kg	16	4	0	0	0	0	0
		30 mg/kg	14	4	0	0	0	0	0
		100 mg/kg	4	1	0	0	0	0	0
VZ	GD 14-17	Control	20	5	0	0	0	0	0
		10 mg/kg	20	5	0	0	0	0	0
		30 mg/kg	20	5	0	0	0	0	0
		100 mg/kg	20	5	17 (5)	17 (5)	1 (1)	1 (1)	0
	GD 18-21	Control	20	5	0	0	0	0	0
		10 mg/kg	16	4	0	0	1 (1)	0	0
		30 mg/kg	16	4	0	0	0	0	0
		100 mg/kg	15	4	0	0	0	0	0
DES	GD 14-17	Control	19	5	0	0	0	0	0
		0.1 μg/kg	12	3	0	0	0	0	0
		1 μg/kg	20	5	0	0	0	0	0
		10 μg/kg	16	4	0	0	0	0	0
		100 μg/kg	16	4	0	0	0	0	0
		300 μg/kg	12	4	0	0	0	0	0
	GD 18-21	Control	16	4	0	0	0	0	0
		0.1 μg/kg	20	5	0	0	0	0	0
		1 μg/kg	16	4	0	0	0	0	0
		10 μg/kg	20	5	0	0	0	0	0
		100 μg/kg	20	5	0	0	0	0	0
		300 μg/kg	7	2	0	0	0	1 (1)	0
EE	GD 14-17	Control	16	4	0	0	0	0	0
		10 μg/kg	16	4	0	0	0	0	0
		100 μg/kg	20	5	0	0	0	0	0
	GD 18-21	Control	19	5	0	0	0	0	0
		10 μg/kg	15	4	0	0	0	0	0
		100 μg/kg	16	4	0	0	0	0	0
TAM	GD 14-17	Control	16	4	0	0	0	0	0
		0.03 mg/kg	15	4	0	0	0	0	0
		0.1 mg/kg	7	2	0	0	0	0	0
	GD 18-21	Control	19	5	0	0	0	0	0
		0.03 mg/kg	12	3	0	0	0	0	0
		0.1 mg/kg	11	3	0	0	0	0	0

Value: number of cases (litters) with abnormality.
FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day.

Cleft prepuce and cleft phallus were also observed in males from dams exposed to 100 mg/kg of VZ on GD 14–17 (Figs. 2C, 2D). The incidence of cleft prepuce and cleft phallus was 85% (17 cases in all of the 5 litters). However, there was no cleft at the prepuce or phallus of males from dams exposed to VZ on GD 18–21 (Figs. 2E, 2F). The time of sexual maturation is determined by complete separation of the prepuce from the ventral surface of the glans penis, but preputial separation could not determined in males with cleft phallus, since complete separation in the glans penis was not evident.

The testis of males from dams exposed to 10 mg/kg of

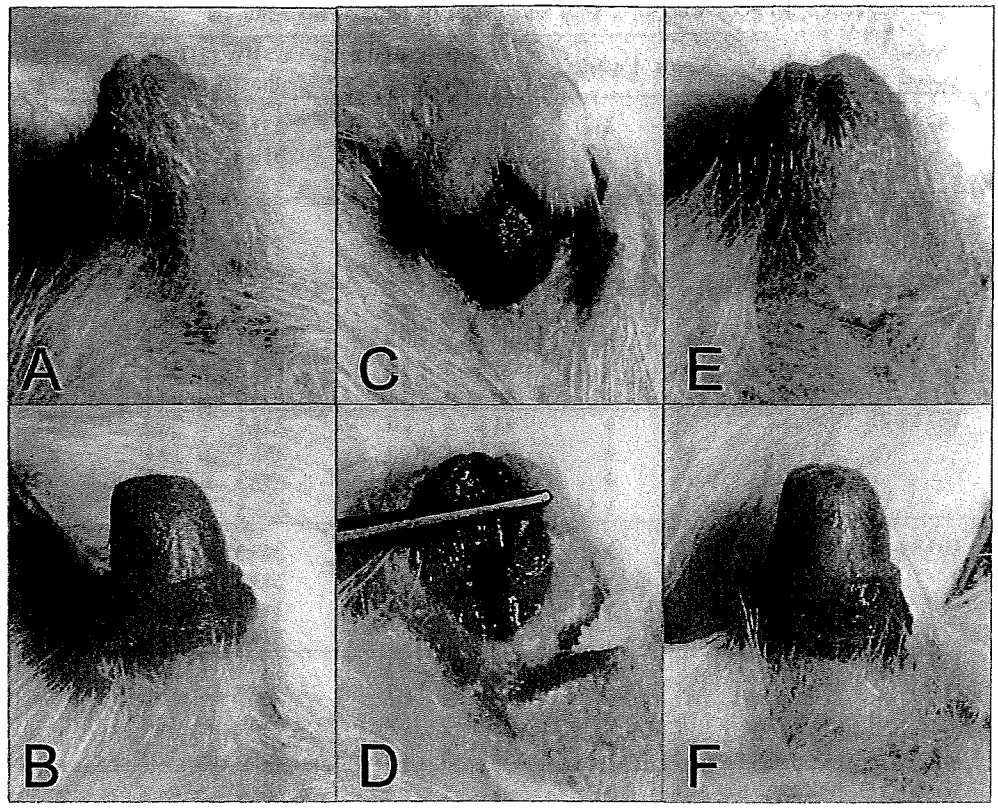

Fig. 2. External genitalia of male rats on PND 56.
A and B: Control male rat. Prepuce is completely retracted. C and D: Male rat from a dam exposed to 100 mg/kg vinclozolin on GD 14–17. Ventral side of the prepuce has a cleft and the glans penis is observed from the cleft (C). The ventral part of the penis is incompletely formed (cleft phallus, shown in D). E and F: Male rat from a dam exposed to 100 mg/kg vinclozolin on GD 18–21. There was no cleft on the ventral surface of the prepuce or penis.

FLU on GD 14–17 (17%, 2 cases in 2 litters) or 100 mg/kg of VZ on GD 14–17 (5%, one case in one litter) did not descend into the scrotum and was located in the ventral subcutis (ectopic testis) instead. One male from a dam exposed to 10 mg/kg VZ on GD 18–21 also had an ectopic testis. Ectopic testis was observed unilaterally. Marked inflammation in the prostate and seminal vesicle occurred in the group exposed to 10 mg/kg FLU on GD 18–21, and 5 of 12 males in this group died from the severe inflammation. Males with marked prostatitis and vesiculitis did not show hypospadias in their glans penis.

3) Preputial separation

Males with hypospadias were excluded from the preputial separation analysis, and the resulting days of preputial separation of males without hypospadias are summarized in Table 2. Preputial separation in males from dams exposed to 10 mg/kg of FLU was significantly delayed in both GD 14–17 and GD 18–21 treatment groups, and 2 males in the GD 18–21 treatment group had incomplete preputial separation on the day of autopsy, PND 56. In the group exposed to 100 mg/kg of DDE on GD 18–21, all pups of 3 litters died. There were no differences among the other 3 groups. In the 100 mg/kg VZ groups, male pups without hypospadias showed no difference from the control group, and the lower dose groups did not show any significant difference from the control. Two of 4 pregnant females administered 300 μg/kg of DES from GD 18 were excluded from the experiment because these females delivered their pups on GD 21 before the end of the administration period. Preputial separation of male offspring exposed to DES was not different from the control group. EE and TAM administered in any exposure period did not affect preputial separation.

Body weight on the day of complete preputial separation was significantly higher in males from dams exposed to FLU on GD 18–21, but there were no changes in other chemically-treated groups.

4) Measurement of AGD on PND 6

Table 3 shows the AGD, body weight, and correction values of AGD [AGD (mm)/ $\sqrt[3]{\text{body weight (g)}}$ of pups] on PND 6. Correction values of AGD were significantly decreased in the groups exposed to 10 mg/kg FLU on GD 14–17 or GD 18–21, and the group exposed to 100 mg/kg VZ on GD 14–17. DES induced a significant reduction in AGD of the 100 and 300 μg/kg groups exposed on GD 18–21. DDE, EE and TAM did not induce a significant

Table 2. Preputial Separation and Body Weights of Male Rats Prenatally Exposed to Chemicals

Chemical	Dosing period	Group	Males	Litters	PND of preputial separation	BW (g) on the day of PPS	Incomplete separation
FLU	GD 14-17	Control	12	3	42.8 ± 2.2	232.2 ± 26.5	0
		1 mg/kg	12	3	42.4 ± 0.3	216.5 ± 3.3	0
		10 mg/kg	5	2	49.1 ± 0.6 **	271.2 ± 41.3	0
	GD 18-21	Control	12	3	43.2 ± 1.4	227.0 ± 19.2	0
		1 mg/kg	12	3	44.5 ± 0.7	249.8 ± 12.7	0
		10 mg/kg	5	2	51.3 ± 0.5 **	322.2 ± 13.7 **	2 (1)
DDE	GD 14-17	Control	12	3	42.8 ± 2.2	232.2 ± 26.5	0
		10 mg/kg	12	3	42.6 ± 0.4	242.1 ± 23.3	0
		30 mg/kg	16	4	43.1 ± 1.0	248.3 ± 20.1	0
		100 mg/kg	16	4	44.6 ± 3.0	242.5 ± 14.0	0
	GD 18-21	Control	14	4	43.8 ± 1.5	231.7 ± 26.7	0
		10 mg/kg	16	4	43.3 ± 1.6	233.7 ± 19.6	0
		30 mg/kg	14	4	43.0 ± 1.0	239.3 ± 16.2	0
		100 mg/kg	4	1	43.5	233.5	0
VZ	GD 14-17	Control	20	5	44.4 ± 1.2	245.3 ± 10.5	0
		10 mg/kg	20	5	43.7 ± 1.6	243.7 ± 16.4	0
		30 mg/kg	20	5	44.5 ± 1.7	246.2 ± 13.7	0
		100 mg/kg	3	1	43.7	254.1	0
	GD 18-21	Control	20	5	44.1 ± 0.3	252.1 ± 36.7	0
		10 mg/kg	16	4	43.8 ± 1.5	248.2 ± 17.3	0
		30 mg/kg	16	4	43.3 ± 1.9	240.8 ± 11.4	0
		100 mg/kg	15	4	45.1 ± 1.7	259.2 ± 22.3	0
DES	GD 14-17	Control	19	5	44.4 ± 1.7	246.4 ± 12.9	0
		0.1 μg/kg	12	3	43.3 ± 0.5	242.3 ± 12.7	0
		1 μg/kg	20	5	43.3 ± 1.3	243.3 ± 17.6	0
		10 μg/kg	16	4	42.8 ± 1.3	244.2 ± 19.9	0
		100 μg/kg	16	4	43.7 ± 1.4	257.9 ± 8.4	0
		300 μg/kg	12	4	46.1 ± 1.0	260.8 ± 20.5	0
	GD 18-21	Control	16	4	44.6 ± 1.1	246.5 ± 16.0	0
		0.1 μg/kg	20	5	45.1 ± 1.1	255.9 ± 22.9	0
		1 μg/kg	16	4	43.3 ± 1.2	252.5 ± 18.5	0
		10 μg/kg	20	5	43.4 ± 1.4	241.6 ± 7.5	0
		100 μg/kg	20	5	44.0 ± 0.6	245.3 ± 9.1	0
		300 μg/kg	7	2	46.8 ± 1.1	239.9 ± 1.7	0
EE	GD 14-17	Control	16	4	44.4 ± 1.0	254.6 ± 11.9	0
		10 μg/kg	16	4	45.6 ± 2.0	240.2 ± 6.2	0
		100 μg/kg	20	5	45.8 ± 2.2	261.6 ± 20.2	0
	GD 18-21	Control	19	5	43.6 ± 1.7	246.6 ± 9.6	0
		10 μg/kg	15	4	43.3 ± 0.5	248.3 ± 10.1	0
		100 μg/kg	16	4	42.8 ± 1.6	247.6 ± 12.3	0
TAM	GD 14-17	Control	16	4	44.4 ± 1.0	254.6 ± 11.9	0
		0.03 mg/kg	15	4	44.5 ± 1.3	252.7 ± 25.2	0
		0.1 mg/kg	7	2	43.5 ± 0.3	244.2 ± 11.6	0
	GD 18-21	Control	19	5	43.6 ± 1.7	246.6 ± 9.6	0
		0.03 mg/kg	12	3	43.2 ± 1.0	242.7 ± 32.3	0
		0.1 mg/kg	11	3	43.7 ± 0.6	257.5 ± 30.3	0

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin, DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day; PND: postnatal day; BW: body weight; PPS: preputial separation.

Incomplete separation: number of animals (litters) with incomplete separation on PND 56.

**: significantly different from control, $p<0.01$.

Table 3. Anogenital Distance (AGD) of PND 6 Male Rats Prenatally Exposed to Chemicals

Chemical	Dosing period	Group	Males	Litters	AGD (mm)	Correction value of AGD (mm/$\sqrt[3]{g}$)	Body weight (g)
FLU	GD 14-17	Control	12	3	7.07 ± 0.59	2.85 ± 0.11	15.4 ± 3.1
		1 mg/kg	20	3	6.21 ± 0.09	2.63 ± 0.03	13.3 ± 1.0
		10 mg/kg	26	3	5.53 ± 0.39 **	2.35 ± 0.12 **	13.0 ± 1.0
	GD 18-21	Control	19	3	6.70 ± 0.25	2.93 ± 0.07	11.9 ± 0.7
		1 mg/kg	20	3	7.04 ± 0.71	2.89 ± 0.21	14.4 ± 1.3
		10 mg/kg	22	3	5.82 ± 0.45	2.42 ± 0.22 *	14.1 ± 1.2
DDE	GD 14-17	Control	12	3	7.07 ± 0.59	2.85 ± 0.11	15.4 ± 3.1
		10 mg/kg	23	3	6.84 ± 0.38	2.84 ± 0.01	14.0 ± 2.2
		30 mg/kg	30	4	6.67 ± 0.59	2.78 ± 0.13	13.8 ± 2.3
		100 mg/kg	23	4	6.32 ± 0.44	2.71 ± 0.11	12.8 ± 1.6
	GD 18-21	Control	19	4	7.55 ± 0.86	3.10 ± 0.20	14.5 ± 2.3
		10 mg/kg	26	4	6.96 ± 0.56	2.93 ± 0.20	13.4 ± 1.1
		30 mg/kg	26	4	7.29 ± 0.45	3.00 ± 0.09	14.5 ± 2.9
		100 mg/kg	5	1	7.40	3.04	14.5
VZ	GD 14-17	Control	35	5	6.75 ± 0.39	2.86 ± 0.14	13.2 ± 0.9
		10 mg/kg	38	5	6.60 ± 0.14	2.72 ± 0.08	14.5 ± 1.9
		30 mg/kg	28	5	6.46 ± 0.22	2.73 ± 0.08	13.2 ± 0.4
		100 mg/kg	34	5	4.87 ± 0.48 **	2.02 ± 0.22 **	14.1 ± 2.2
	GD 18-21	Control	43	5	6.25 ± 0.75	2.65 ± 0.29	13.2 ± 2.0
		10 mg/kg	28	4	6.14 ± 0.38	2.59 ± 0.15	13.3 ± 0.8
		30 mg/kg	24	4	6.59 ± 0.31	2.70 ± 0.06	14.7 ± 1.8
		100 mg/kg	26	4	5.74 ± 0.54	2.41 ± 0.20	13.6 ± 1.4
DES	GD 14-17	Control	36	5	6.25 ± 0.41	2.66 ± 0.06	13.0 ± 1.9
		0.1 μg/kg	20	3	6.35 ± 0.13	2.60 ± 0.09	14.6 ± 0.8
		1 μg/kg	45	5	6.35 ± 0.20	2.66 ± 0.06	13.8 ± 1.7
		10 μg/kg	21	4	6.64 ± 0.54	2.75 ± 0.14	14.1 ± 1.8
		100 μg/kg	26	4	6.40 ± 0.62	2.63 ± 0.19	14.4 ± 1.3
		300 μg/kg	17	4	5.77 ± 0.83	2.54 ± 0.30	11.6 ± 0.9
	GD 18-21	Control	26	4	6.35 ± 0.46	2.68 ± 0.15	13.3 ± 0.9
		0.1 μg/kg	44	5	6.22 ± 0.26	2.64 ± 0.09	13.2 ± 1.4
		1 μg/kg	35	4	6.28 ± 0.28	2.63 ± 0.06	13.7 ± 2.2
		10 μg/kg	36	5	6.17 ± 0.31	2.62 ± 0.09	13.1 ± 0.9
		100 μg/kg	27	5	5.47 ± 0.44 **	2.38 ± 0.10 **	12.2 ± 1.6
		300 μg/kg	9	2	4.45 ± 0.35 **	2.08 ± 0.16 **	9.7 ± 0.0
EE	GD 14-17	Control	30	4	6.73 ± 0.60	2.82 ± 0.13	13.6 ± 2.1
		10 μg/kg	26	4	6.16 ± 0.52	2.76 ± 0.15	11.1 ± 1.2
		100 μg/kg	37	5	6.58 ± 0.19	2.80 ± 0.07	13.0 ± 1.0
	GD 18-21	Control	32	5	6.78 ± 0.41	2.81 ± 0.15	14.1 ± 0.6
		10 μg/kg	35	4	6.98 ± 0.34	3.00 ± 0.16	12.6 ± 0.5
		100 μg/kg	31	5	7.07 ± 0.54	2.89 ± 0.22	14.7 ± 1.5
TAM	GD 14-17	Control	30	4	6.73 ± 0.60	2.82 ± 0.13	13.6 ± 2.1
		0.03 mg/kg	25	4	6.50 ± 0.88	2.71 ± 0.21	13.8 ± 2.7
		0.1 mg/kg	9	2	6.52 ± 0.19	2.73 ± 0.02	13.7 ± 0.9
	GD 18-21	Control	32	5	6.78 ± 0.41	2.81 ± 0.15	14.1 ± 0.6
		0.03 mg/kg	16	3	6.84 ± 0.06	2.73 ± 0.12	15.8 ± 1.7
		0.1 mg/kg	25	4	6.57 ± 0.46	2.68 ± 0.14	15.0 ± 2.6

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day; PND: postnatal day.

Correction value of AGD: AGD(mm)/$\sqrt[3]{\text{body weight (g)}}$.

*: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.