表 2 選択したアミノ酸
Table 2 Selected amino acids.

β_1 アドレナリン受容体	W117	T118	D121	V122	A208	S211	S215	W303	F306	F307	N310	N329	Y333							
β_2 アドレナリン受容体	W109	D113	V114	V117	T118	Y199	A200	S203	S204	S207	W286	F289	F290	N293	Y308	N312	Y316			
アデノシン受容体	L85	F168	E169	M177	N181	W246	L249	H250	N253	M270	Y271	I274								
ロドプシン	Y43	M44	L47	T94	E113	A117	T118	G120	G121	E122	M207	H211	F212	F261	W265	Y268	A269	F293	A295	K296

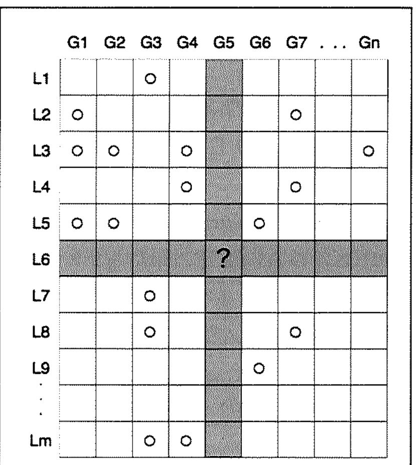

図 3 リガンドと GPCR 両者についての情報が全て欠けている予測の例．また，正例のみのデータセットの例．
Fig. 3 An example of prediction with neither known ligands nor known GPCRs. And also the example of the data set containing only positive samples.

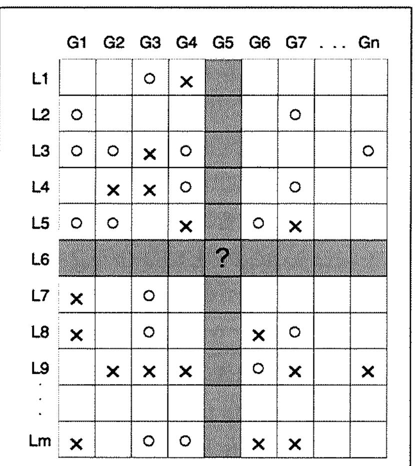

図 4 負例生成済みデータセット．× は負の相互作用を表す．網掛け部分は G5 に対して全てのリガンドとの相互作用が未知であることと，L6 に対して全ての GPCR との相互作用が未知であることを意味している．網掛け部分が交差する部分が double orphan であり，2-way prediction 法の予測する対象である．
Fig. 4 An example of the data set containing generated negative samples. The × represent negative interactions. The information in shaded area is absent. The crossing position is the double orphan, which is the target of the 2-way prediction method.

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	G1	G2	G3	G4	G5	G6	G7	...	Gn
L1			○	×					
L2	○						○		
L3	○	○	×	○					○
L4		×	×	○			○		
L5	○	○		×		○	×		
L6	○	×	○	×	?	○	×		
L7	×		○						
L8	×		○			×	○		
L9		×	×	×		○	×		×
⋮									
Lm	×		○	○		×	×		

図 5 2-way prediction 法による予測．G1，G2，G3，などについて，L6 以外のリガンドの特徴を用いて学習し，L6 の行を予測値で埋めた例．L6 の行の予測値を用いて，最終的に?の部分を予測する．

Fig. 5 Prediction by the 2-way prediction method. The interaction of GPCRs (except G5) with L6 is predicted with the training data set except L6. By using the predicted interaction of L6 with GPCRs, the interaction between G5 and L6 is determined.

学習できないため，今回は正例が 5 以上ある 62 個の GPCR に対して予測実験を行った．予測にはサポートベクターマシン（SVM）を利用した．SVM を利用する際には，種々のカーネルの使用が考えられるが，今回は線形カーネル，多項式カーネル，ガウスカーネル，の 3 種類のカーネルと，各パラメータを変化させ予測精度の比較を行い，最もよい精度をその GPCR における予測精度とした．予測精度は以下で定義される Accuracy を使用し，5-fold のクロスバリデーションによって精度を計算した．

$$\mathrm{Accuracy} = \frac{\text{正負を正しく予測できたサンプル数}}{\text{全てのサンプル数}}$$

- 方法（2）

  各リガンドに対して，相互作用既知 GPCR の特徴を用いて未知 GPCR との相互作用を予測する．予測方法は方法（1）の場合と同様に行い，正例が 5 以上ある 83 個のリガンドに対して予測実験を行った．SVM の利用や，カーネルや各パラメータの選択方法も方法（1）と同様にし，予測精度も同様に計算した．

- 方法（3）

  2-way prediction 法（A）と 2-way prediction 法（B）はどちらも手順は似ているので，2-way prediction 法（A）の手順を以下に述べる．まず，すでに正例か負例かが分かっているデータに対して，その GPCR 以外で，安定した予測結果を得るために正例を 10 以上（$k = 10$）含んでいる GPCR を選ぶ（48 個）．選ばれた GPCR それぞれに対して，方法（1）と同じようにクロスバリデーションを行い，最も高い精度を得られたカーネルとパラメータを利用して，orphan リガンドの予測を行う．その結果，orphan リガンドに対して，複数の GPCR に対して相互作用するかどうかという複数のデータが得られる．orphan リガンドの相互作用を予測した後，GPCR の特徴と得られた予測結果を用いて，クロスバリデーションを行い最も高い精度を得られたカーネルとパラメータを用いて，orphan GPCR との相互作用の予測を行う．2-way prediction 法の予測性能を評価するために，正の double orphan 50，負の double orphan 50 を仮定し最終的に 2-way prediction 法による予測精度を計算する．

4.2 結　　果

- 方法（1）の実験結果．サンプル数の違いによる予測精度を表 3 に示す．この表 3 は辺 4 から 9，サポート値 61 から 70 の場合である．ここでは 5 から 200 以上の正例を含む GPCR の精度の平均を計算した．予測精度にあまり変化はなく，学習リガンド数にあまり依存しないと考えられる．5 より小さい場合に関しては，リガンドが少なすぎ，予測精度に大きなばらつきがあったため今回は 5 以上を比較している．

  特徴として部分グラフの取り方のサポート値を変化させた性能の変化を図 6 に示す．棒グラフは各サポート値の範囲に対する予測精度を表しており，折れ線グラフは各サポート値に含まれる特徴数（部分グラフ数）である．部分グラフの総数は非常に多く，特徴としてすべての部分グラフを利用することは難しいため，サポート値と辺の数に範囲を設定し，その範囲に含まれる部分グラフのみを特徴として利用した．辺の数は 4 から 9 の範囲を設定し，それぞれについてサポート値を 31 から 100 まで 10 刻みで範囲を設定した．その結果が図 6 である．

  サポート値の違いによる予測精度の違いは大きくても 1%前後であり，あまり変化がなかったといえる．サポート値を小さくすると，部分グラフの数が大きくなりすぎ，特徴がうまくとれない．逆にサポート値を大きくすると，ほぼ全てのリガンドに含まれる一般的すぎる部分グラフとなり，予測の役に立たない．そのため中程度のサポート値を選んでいるが，中程度のサポートを選んだ場合には，あまり大きな予測精度の差はなかっ

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たことがわかる．すなわち，中程度のサポート値を持つ部分グラフのもつ特徴に大きな差がないことになる．

- 方法（2）の実験結果．

GPCR 数の違いによる予測精度の変化を表 4 に示す．今回のデータセットでは各リガンドに相互作用する GPCR の数が少ないので 5 から 20 以上の GPCR を含むリガンドのみの性能の平均を計算した．あまり違いがみられないため，学習 GPCR 数にはあまり依存しないことがわかる．リガンドの情報を用いたときと同様に，5 より小さい場合には予測精度におおきなばらつきがあったため，5 以上のサンプルを含むリガンドのみを比較した．

GPCR の特徴であるリガンド結合部位の取り方の種類を変え，その違いを表 5 に示す．今回リガンド結合部位と予測される部分として 6 種類のリガンド結合部位を比較した．β_1 アドレナリン受容体（adreβ_1），β_2 アドレナリン受容体（adreβ_2），アデノシン受容体（adeno），ロドプシン（rho），のそれぞれのリガンド結合部位に加え，2 種類のアドレナリンレセプタのリガンド結合部位の和集合（adre），Jacob ら[3]（Jacob）と同じリガンド結合部位を比較した．2 種類のアドレナリンレセプタの和集合，アデノシンレセプタの結合部位の予測精度がよくなっている．β_1 アドレナリンレセプタのみの場合には予測精度が下がっているが，このレセプタがヒトではなく七面鳥のものであり，今回対象としている GPCR がヒトであることを考えると納得できる．

- 方法（3）の実験結果．

リガンドの特徴の取り方と GPCR の特徴の取り方を変えて実験を行った結果を表 6，表 7 に示す．表 6 は，各 GPCR に関して orphan リガンドの予測を先に行った場合であり，表 7 は，各リガンドに関して orphan GPCR の予測を先に行った場合である．ともに辺の数は 6 から 10 の場合であり，サポート値の違いによる予測精度を示している．各リガンドに関して orphan GPCR の予測を先に行った場合はランダム予測の場合（50%）とほぼ同等であり，この方法は実用的でないことがわかる．理由として推測されることは，各リガンドについて相互作用する GPCR の数が十分に存在するものが少ないためと考えられる．この数が少ないため，2 段階目に利用するための予測値の数が少なくなる．すると誤って予測してしまった結果に影響されやすくなり，最終的な精度があがらないと考えられる．逆に，各 GPCR に関して orphan リガンドの予測を先に行った場合の予測は，よい場合で 70%を超えて，ある程度予測に成功している．

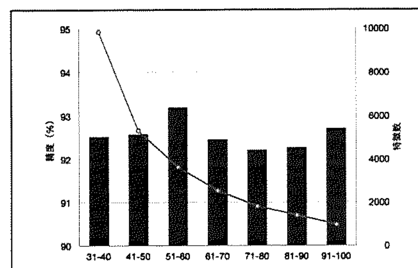

図 6 辺の数の範囲を 4 から 9 に設定したときの，サポート値の変化による予測精度の変化．左の縦軸は予測精度（%）であり棒グラフに対応している．右の縦軸は特徴数であり折れ線グラフに対応している．横軸は設定したサポート値の範囲である．

Fig. 6 Dependence of the prediction accuracy on the support value. The number of edges is set between 4 and 9. The vertical axes on the right and left sides correspond to the number of features for the line graph and the prediction accuracy for the bar graphs (%), respectively. The horizontal axis corresponds to the support value.

4.3 考　　察

リガンド情報を用いて各 GPCR に関して未知リガンドを予測する方法はリガンドが 5 つ以上ある場合には平均で 93%となり予測に成功している．また，リガンドが 5 つ以上ある GPCR は全体の 77.5%であり，今回はサポート値や辺の数によって特徴を選択したが，こ

表 3 GPCR のリガンド数の違いによる予測精度（%）の変化．
Table 3 Prediction accuracy with different number of GPCRs.

リガンド数	5	10	20	50	100	200
予測精度	93.1	92.4	91.9	91.4	91.4	92.6
リガンド数以上の正例を含む GPCR 数	62	48	41	38	10	4

表 4 GPCR 数の違いによる予測精度（%）の変化．
Table 4 Prediction accuracy with different number of ligands.

GPCR 数	5	10	15	20	30
予測精度	97.0	94.0	94.4	95.7	96.6
GPCR 数以上の正例を含むリガンド数	83	13	6	3	1

表 5 相互作用既知の GPCR が 5 以上存在するリガンドについて，リガンド結合部位の違いによる予測精度の変化．
Table 5 Prediction accuracy with different ligand binding sites.

結合部位	adre	adreβ_1	adreβ_2	adeno	rho	Jacob
予測精度	94.4	90.5	93.1	94.4	93.3	92.1

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の選択の違いによって大きな予測精度の変化はみられなかった.

GPCR の情報を用いて各リガンドに関して未知 GPCR を予測する方法は，GPCR が 5 つ以上ある場合には平均で約 97%となり予測に成功している．しかし，GPCR が 5 つ未満のリガンドは多く，全体の 96.5%である．GPCR の情報が豊富に存在する場合には非常に有効な方法であることが分かったが，実際には GPCR の情報が少ない場合が多く，この方法を適用できない場合もある．十分にデータが存在し，予測が可能な場合には，リガンド結合部位の選び方によって予測精度が変わことが分かった．このことから，リガンド結合部位は結合するリガンドに関係しており，リガンド結合部位の定義がリガンド予測に重要であることを示唆している.

また，2-way prediction 法では，リガンド，GPCR，両方の情報が無い場合に，最大で精度が 72%となり，全く情報の無い相互作用であっても，予測可能であることが分かる.

Jacob ら[3] も GPCR とリガンドの相互作用の予測を SVM によって行っており，この研究では彼らと同じデータセットを使ったので，性能の比較を行った．今回使った 3 つの方法について比較した．その比較をまとめた表を表 8 に示した.

Jacob らはリガンド予測の際に他の GPCR からの情報も使っているが，我々は他の GPCR からの情報は全く使っていない．それにも関わらず，ほぼ同等の性能を発揮することができており，我々の特徴の取り方が有効であることが言える．また，我々は一般的なカーネルのみを利用していることから，複雑なカーネルなどの設計もそれほど意味がないことも分かる．orphan GPCR の予測については，我々は他の GPCR に相互作用するリガンドの情報が豊富にある場合には予測に成功しているが，ない場合には予測自体が不可能である．よってこのようなあるリガンドに相互作用する GPCR の情報が少ないデータセットでは不利になってしまう．他の GPCR に相互作用するリガンドがある場合において，orphan GPCR の予測は，Jacob らの方法に比べて我々の方法は非常に有効であると言える．リガンド情報がない場合にも我々は 2-way prediction によって予測を可能にしている．Jacob らはこの最も難しい問題についての結果を出していないため直接比較することはできなかった.

擬似的に比較をするため以下の方法をとった．Jacob らと同様に orphan GPCR を予測する場合に，double orphan となる相互作用は 1723 あり，全体の相互作用数に対する割合は 70.4%である．すなわち，70.4%は 2-way prediction 法の予測の対象となる．残りの 29.6%の相互作用については，Jacob らは予測するリガンドと同じリガンドを学習に利用したことになる．orphan GPCR に相互作用するリガンドを予測する場合に，他の GPCR に相互作用するリガンドが分かっている場合，我々は方法（2）によって予測する．2-way prediction の予測精度を 72%，方法（2）の予測精度を 97%として，予測精度の重み付き平均を求めると，$72 \times 0.704 + 97 \times 0.296 = 79.6\%$となり，Jacob らとわずかではあるがよい精度が期待できる.

表 6 orphan リガンド予測を先に行った場合の 2-way prediction 法よる予測の結果（%）.
Table 6 Prediction accuracy with 2-way prediction. In this prediction, orphan ligands are predicted first.

サポート値	adre	adreβ_1	adreβ_2	adeno	rho	Jacob
61-70	69	66	70	62	59	68
81-90	69	71	72	65	66	61

表 7 orphan GPCR 予測を先に行った場合の 2-way prediction 法よる予測の結果（%）
Table 7 Prediction accuracy with 2-way prediction. In this prediction, orphan GPCRs are predicted first.

サポート値	adre	adreβ_1	adreβ_2	adeno	rho	Jacob
61-70	47	55	42	44	51	48
81-90	40	50	54	45	47	48

5. おわりに

方法 (1)，方法 (2) によって，学習サンプルが豊富に存在する場合には非常に高い精度で予測できることを示した．情報の少ない GPCR の問題に対応するために，方法 (3) である 2-way prediction 法を開発し，全くの情報の無い GPCR とリガンドに対する予測が可能であることを示した．GPCR リガンドの予測の問題は情報が少なく非常に難しく，まだ改善するべき部分は多くある.

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表 8 既存研究との比較
Table 8 The comparison with the previous research

	リガンドの予測	GPCR の予測	全ての情報がないときの予測
Ours	93	97	72
Jacob	93	78	

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Comparative Contribution of the Aryl Hydrocarbon Receptor Gene to Perinatal Stage Development and Dioxin-Induced Toxicity Between the Urogenital Complex and Testis in the Mouse[1]

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ABSTRACT

TCDD (2,3,7,8-tetrachlorodebenzo-*p*-dioxin) requires the presence of the aryl hydrocarbon receptor (*Ahr*) gene for its toxic effects, such as reproductive disorders in male offspring of maternally exposed rats and mice. To study the involvement of the *Ahr* gene in producing the toxic phenotype with respect to testicular development, we administered a relatively high dose of TCDD to mice with three different maternally derived *Ahr* genotypic traits, and then compared several *Ahr*-dependent alterations among male reproductive systems on Postnatal Day 14. Reduction in anogenital distance and expression of prostatic epithelial genes in the urogenital complex (UGC) were detected in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice exposed to TCDD, whereas no difference was observed in $Ahr^{-/-}$ mice. In situ hybridization revealed the absence of probasin mRNA expression in the prostate epithelium, despite the obvious development of prostatic lobes in TCDD-exposed mice. In contrast to obvious prostatic dysfunction and induction of cytochrome P450 (CYP) family genes in the UGC by TCDD, no alterations in testicular functions were observed in germ cell/Sertoli cell/interstitial cell marker gene expression or CYP family induction. No histopathological changes were observed among the three genotypes and between control and TCDD-exposed mice. Therefore, mouse external genitalia and prostatic development are much more sensitive to TCDD treatment than testis. Further, the *Ahr* gene, analyzed in this study, does not significantly contribute to testicular function during perinatal and immature stages, and the developing mouse testis appears to be quite resistant to TCDD exposure.

aryl hydrocarbon receptor, developmental biology, dioxin, knockout mouse, prostate, spermatogenesis, testis, toxicology

INTRODUCTION

TCDD (2,3,7,8-tetrachlorodebenzo-*p*-dioxin) is an extremely potent xenobiotic chemical. Maternal exposure to TCDD induces a wide range of physiological alterations and toxicities in the fetus and pups of laboratory animals and perhaps in humans [1]. TCDD induces various toxicological endpoints in male reproductive organs, such as decreased size of sex-accessory glands and reduced sperm counts in testis, epididymis, and ejaculate [2–10]. Although male rat and mouse offspring exposed to TCDD in utero can produce testicular androgen normally, the androgen responsiveness of the ventral prostate is lowered by unknown mechanisms of TCDD [9–12].

Interestingly, the effects of TCDD on testicular development and perinatal stage spermatogenesis are still unclear because the results are contradictory [2–10]. Some studies on testicular development that used conventional experimental animals reported the presence of slight reductions in testicular weight, daily sperm production, and steroidogenesis [2–5]. On the other hand, clear negative data concerning the TCDD effect on testicular development were presented in many other papers [7–10]. Taken together, the impairment in prostate development by in utero TCDD exposure appears to occur in many mammals, including rats and mice, but it is not understood whether or how susceptible the testicular development and perinatal stage spermatogenesis is to the TCDD exposure.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mostly mediates inductions of drug-metabolizing enzymes such as members of the CYP1A family, and it appears to act as a sensor for environmental contaminants such as dioxins and polyaromatic hydrocarbons [13, 14]. Experimental studies with *Ahr* gene knockout mice have already revealed that AHR plays an essential role in the occurrence of multiple TCDD-induced adverse effects, including teratogenic cleft palate and hydronephrosis [15, 16]. Reproductive disorders, such as prostatic growth impairment in male offspring following maternal exposure to TCDD, were also shown to be dependent on the *Ahr* gene [17].

Some evidence also suggested that AHR was involved in developmental signaling in the immune and hepatic systems [18, 19]. In analyses using *Ahr*-null female mice, AHR was

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also required for normal ovarian germ cell dynamics, based on the observation that numbers of nonatretic primordial, primary, and small preantral follicles in ovaries of *Ahr*-null females at early postpartum stages were higher than those in wild-type mice [20–22]. Moreover, AHR cooperates with an orphan nuclear receptor, NR5A1 (also known as Ad4BP/SF-1), to activate P450 aromatase (CYP19) gene transcription in ovarian granulosa cells and modulate endogenous estrogen production in the female reproductive cycle [23]. AHR is also expressed in interstitial cells and male germ cells at specific stages [24]. More recently, AHR has been demonstrated to have a ubiquitin ligase activity, which enhances the degradation of estrogen and androgen receptors, suggesting that AHR may modulate androgen sensitivity in normal development [25]. To date, the possible involvement of AHR in testicular development during immature stages using AHR agonist administration or *Ahr*-null male mice has not been addressed.

To the best of our knowledge, this is the first study that used *Ahr*-null mice to investigate the involvement of the *Ahr* gene in early stages of testicular development and to assess the differences in susceptibility to in utero TCDD exposure between prostate and testicular development.

MATERIALS AND METHODS

Materials

TCDD was purchased from Cambridge Isotope Laboratory (Andover, MA). The purity was higher than 99.5%. Corn oil for dissolving TCDD or the control vehicle was obtained from Sigma-Aldrich (St. Louis, MO). TRIzol reagent, SuperScript III RNase H- Reverse Transcriptase, and oligo(dT)12–18 primer were purchased from Invitrogen (Carlsbad, CA). SYBR Premix Ex Taq (Perfect Real Time) was purchased from TAKARA BIO, Inc. (Otsu, Japan). The plasmid pGEM-TEasy vector was obtained from Promega Corp. (Madison, WI).

Animals and TCDD Administration

Ahr knockout mice were kindly provided by Dr. Yoshiaki Fujii-Kuriyama (Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba) [15]. They were bred in our own facility at the National Institute for Environmental Studies (NIES). All described procedures were approved by the NIES Institutional Animal Care and Use Committee and were performed in accordance with the Guidelines for Animal Experiments at the NIES. They were maintained in a controlled environment of temperature 24 ± 1°C, humidity 45 ± 5%, and a 12L:12D cycle and were given food and distilled water ad libitum. *Ahr* heterozygous male mice were back-crossed with wild-type female C57BL/6J mice (CLEA Japan, Tokyo, Japan) six times, and heterozygous offspring of both sexes were used in this study. The heterozygous female mice (7- to 10-wk-old) were mated 1:1 with the heterozygous males overnight, and the females that had a vaginal plug on the following morning were designated as being pregnant at Gestational Day 0 (GD0). Dams were housed individually in clear plastic cages with heat-treated wood chips as bedding. On GD13, pregnant mice were given a single dose of TCDD orally (10 μg/kg body weight, close to a lethal dose for a C57BL/6J fetus) or an equivalent volume of vehicle (95% corn oil, 4% n-nonane; 5 ml/kg) as control. Male pups were killed under diethyl ether anesthesia on Postnatal Day 14 (PND14).

Sample Collection

On PND14, immediately before euthanization, the anogenital distance, determined by the length from the base of the genital tubercle to the anterior edge of the anus, was measured with a digital caliper. We also measured the crown-anal length (the distance between the nose and anterior edge of the anus). The testis and epididymis on both sides were excised from the abdomen, and the surrounding adipose tissue was carefully removed. After removing urine from the bladder, the deferent ducts were cut at the base of the bladder. The urogenital complex (UGC), which is a small mass comprising all the lobes of the prostate and seminal vesicle, was then collected by cutting the anterior end of the urethra. All tissue samples were frozen in liquid nitrogen immediately after dissection and kept at −80°C until RNA extraction.

Real-Time RT-PCR

The protocols for real-time RT-PCR quantifications were described previously [26]. Briefly, total RNA was extracted from the UGC (n = 5) and testis (n = 3) using TRIzol reagent. RNA samples were reverse-transcribed with SuperScript III reverse transcriptase and oligo(dT)12–18 primer. Nineteen genes examined in this study: aryl hydrocarbon receptor (*Ahr*), cytochrome P450 1A1 (*Cyp1a1*), cytochrome P450 1A2 (*Cyp1a2*), cytochrome P450 1B1 (*Cyp1b1*), androgen receptor (*Ar*), steroid 5α-reductase type 1 (*Srd5a1*), steroid 5α-reductase type 2 (*Srd5a2*), probasin (*Pbsn*), Mp25 (*Sbp*), PSP94 (*Msmb*), calnexin-t (*Clgn*), Hsp70.2 (*Hspa2*), androgen-binding protein (*Shbg*), cytochrome P450 side chain cleavage (*Cyp11a1*), cytochrome P450 17α/C_{17-20} lyase (*Cyp17a1*), 3β-hydroxysteroid dehydrogenase type I (*Hsd3b1*), 3α-hydroxysteroid dehydrogenase type I (*Akr1c4*), 17β-hydroxysteroid dehydrogenase type III (*Hsd17b3*), and cyclophilin B (*Ppib*). All primer sets are shown in Supplemental Table S1 (available online at www.biolreprod.org). For the real-time RT-PCR, target genes were amplified with SYBR Premix Ex Taq (Perfect Real Time) system by using a LightCycler (Roche, Manheim, Germany). The relative expression level was calculated by normalizing with the average value of each control wild-type ($Ahr^{+/+}$) group. To determine the sequences, the PCR product for each gene was subcloned into pGEM-TEasy vectors or directly sequenced by the dideoxynucleotide chain termination method using the ABI Prism BigDye terminator cycle sequencing kit (PE-Biosystems, Foster City, CA).

In Situ Hybridization

Mouse probasin mRNA was detected in the UGC specimens by in situ hybridization. The UGCs were fixed with neutralized formalin for 48 h, embedded in paraffin, and then cut into 4-μm sections. A template was amplified from the pGEM-TEasy vector inserted with a 377-bp probasin (*Pbsn*) RT-PCR fragment by T7 and SP6 primers to generate sense and antisense transcripts. Digoxigenin-labeled riboprobes were used, and the hybridization was performed using an automated in situ hybridization instrument Gen II (Ventana Medical System, Tucson, AZ). Detection and counterstaining were done with the BlueMap Kit (Ventana Medical System) and Nuclear Fast Red (Sigma-Aldrich).

Immunohistochemistry

Anti-calnexin-t (CLGN), a male germ cell developmental stage-specific protein, was immunostained by the method described previously [27]. Briefly, testes (n = 3; left side) were fixed with Bouin solution and embedded in paraffin. Two different cross-sectional regions (4-μm thickness) from one testis were obtained (six sections from each group). Deparaffinized sections were incubated with anti-mouse calnexin-t antibody, followed by incubation with peroxidase-conjugated goat anti-mouse or rabbit IgG. After washing with PBS, immunoreactivity was detected with diaminobenzidine, followed by hematoxylin counterstaining. Morphometric measurement of the amount of CLGN-positive germ cells was carried out using AxioVision version 4.5 software (Carl Zeiss Co., Ltd., Oberkochen, Germany). The total area of seminiferous tubules within the 1-mm^2 area of the cross-sections from each genotype and treatment group was traced and summed. Then, the CLGN-positive cell numbers were counted and divided by the total area traced for the seminiferous tubules.

Testosterone Assay

Testicular testosterone levels were determined by the enzyme immunoassay (EIA) Kit (Cayman Chemical Co., Ann Arbor, MI). The frozen testis was homogenized in PBS, and the protein concentration was measured by the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). The homogenate was then extracted with diethyl ether, and the ether phase was air-dried. The dried lipophilic substances were resuspended in the appropriate volume of EIA buffer, and the measurements were done according to the manufacturer's instructions.

Statistical Analysis

For statistical analysis, StatView for Windows version 5.0 (SAS Institute, Cary, NC) was used. All data were expressed relative to the means of the control groups. All results are represented as the mean ± SE. Two-way ANOVA was used for comparison of a given parameter among three control groups ($Ahr^{+/+}$, $Ahr^{+/-}$, $Ahr^{-/-}$), followed by the Fisher PLSD post hoc test. $P < 0.05$ was considered significant.

TABLE 1. Reproductive outcomes of male mice exposed to TCDD in utero.[a]

Parameter	Genotype		
	$Ahr^{+/+}$	$Ahr^{+/-}$	$Ahr^{-/-}$
No. of male pups			
Control (n)[b,c]	17 (1.70)	14 (1.40)	6 (0.60)
TCDD (n)[b,d]	6 (0.40)	16 (1.07)	9 (0.60)
Body weight (g)			
Control	6.82 ± 0.41	7.16 ± 0.23	6.04 ± 0.73
TCDD	5.49 ± 0.59	6.59 ± 0.55	7.55 ± 0.27
Crown-anal length (mm)			
Control	54.1 ± 1.2	55.7 ± 0.9	53.0 ± 2.2
TCDD	50.4 ± 2.0	53.1 ± 2.0	56.3 ± 0.9
Anogenital distance (mm)			
Control	3.82 ± 0.11	4.05 ± 0.19	3.66 ± 0.31
TCDD	3.19 ± 0.28*	3.27 ± 0.12**	3.33 ± 0.09
Testicular testosterone level (pg/mg protein)			
Control	140 ± 72 (n = 8)	173 ± 112 (n = 8)	141 ± 86 (n = 5)
TCDD	179 ± 73 (n = 5)	124 ± 33 (n = 8)	197 ± 126 (n = 5)

[a] Data are expressed as means ± SEM, and significant differences were analyzed with ANOVA followed by Fisher PLSD test (versus control of the same genotype, $*P < 0.05$, $**P < 0.01$).
[b] n = The number of male pups per litter.
[c] The number of dams = 10.
[d] The number of dams = 15.

RESULTS

Reproductive Outcome

The number, body weight, and crown-anal length of male pups in each genotype on PND14 are represented in Table 1. There were no statistically significant differences in body weight or crown-anal length among the three genotypes, or between control and TCDD-exposed groups. The number of $Ahr^{+/+}$ male pups exposed to TCDD was only six, which was much lower than the number of control pups, probably due to the fetal death by TCDD exposure in this genotype. Anogenital distance of $Ahr^{+/+}$ and $Ahr^{+/-}$ male pups in the TCDD-exposed groups was significantly reduced compared to control groups ($P = 0.016$ and $P = 0.001$, respectively). In contrast, the anogenital distance of $Ahr^{-/-}$ mice was not significantly different between TCDD-exposed and control mice. ANOVA did not reveal any significant differences in the mean anogenital distance among the three genotypes.

Gene Expressions in UGC

Quantitative RT-PCR analysis of the UGC on PND14 showed that *Ahr* mRNA was detected in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice but not in the $Ahr^{-/-}$ mice. The expression level in the $Ahr^{+/+}$ group was 2-fold higher than that in the $Ahr^{+/-}$ mice, suggesting *Ahr* is transcribed from both alleles in the wild-type mice (Fig. 1). *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* mRNAs, biomarkers of dioxin exposure, were not induced by TCDD

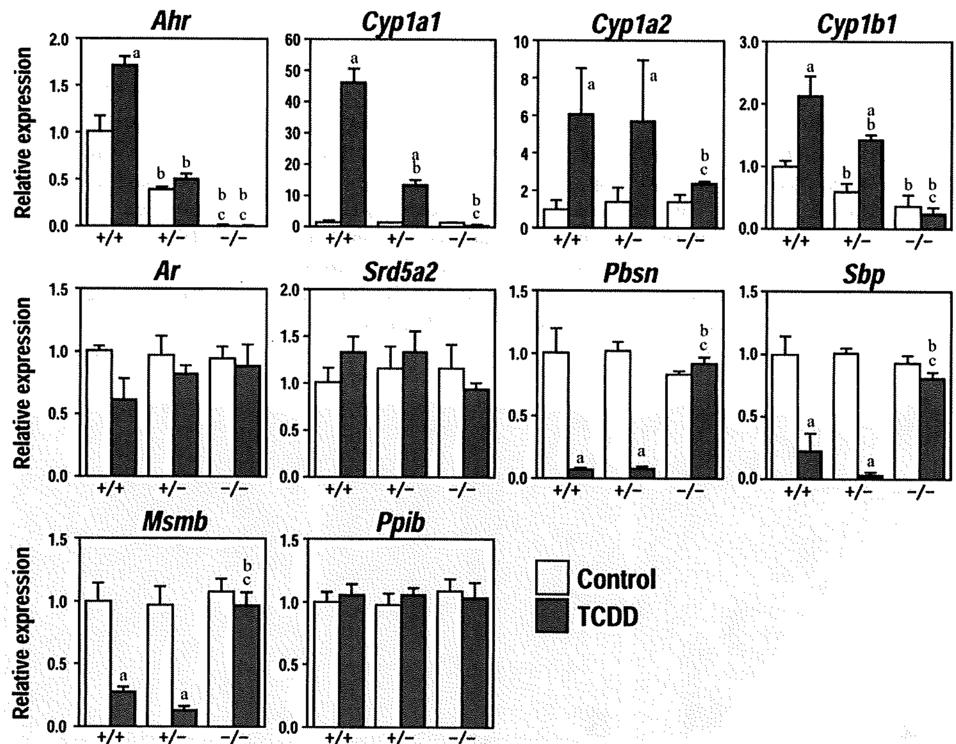

FIG. 1. Quantitative RT-PCR analysis of gene expressions in the urogenital complex of male mouse offspring of three *Ahr* genotypes ($Ahr^{+/+}$, $Ahr^{+/-}$, and $Ahr^{-/-}$) on PND14 with or without TCDD exposure in utero. The values are expressed as mean ± SE for three samples from each group. Note that in utero and lactational exposure to TCDD significantly upregulated *Cyp1a1* and *Cyp1b1* in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice, but not in $Ahr^{-/-}$ mice and that it completely suppressed mRNA expression of the prostatic secretory protein markers *Pbsn*, *Sbp*, and *Msmb* in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice. Significant differences were analyzed with ANOVA followed by the Fisher PLSD test (a, versus control of the same genotype; b, versus the same treatment of wild type; c, versus the same treatment of heterozygous; $P < 0.05$).

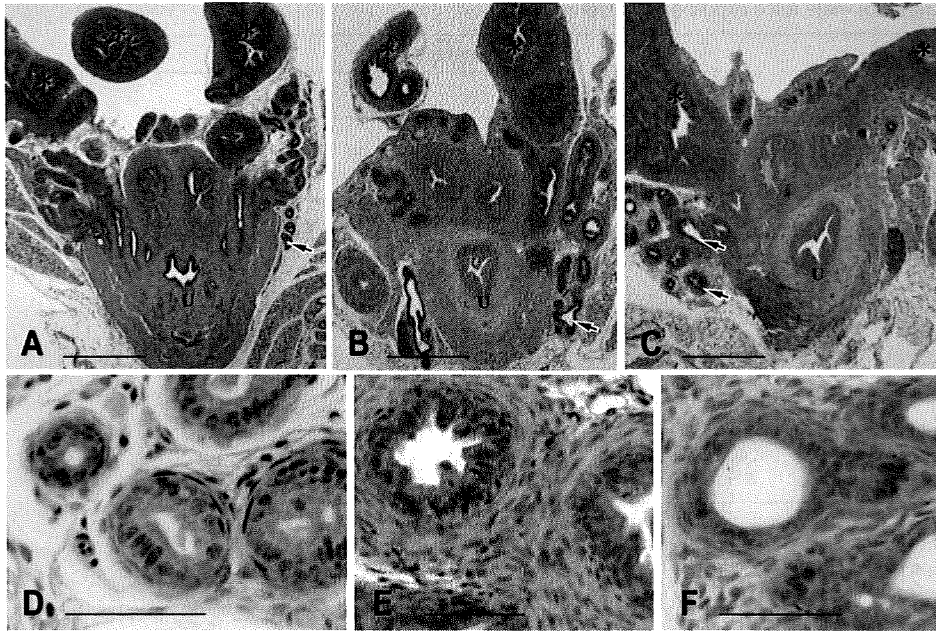

FIG. 2. Histological examinations of the UGC in male mouse pups on PND14. Hematoxylin-eosin staining of $Ahr^{+/+}$ UGCs (**A** and **D**, control; **B**, **C**, **E**, and **F**, TCDD-exposed). Note that control mice had well-developed prostatic lobes on PND14 (**A**). Fewer layers of epithelial cells and increased cell numbers in mesenchymal cells were observed in the dorsolateral prostate lobe of TCDD-exposed animals compared to controls (**E** and **F**). Asterisks, seminal vesicle; arrows, dorsolateral prostate; U, urethra. Bars = 500 μm (**A**, **B**, and **C**), 50 μm (**D**, **E**, and **F**).

in the $Ahr^{-/-}$ mice, but were significantly upregulated in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice. In $Ahr^{+/+}$ and $Ahr^{+/-}$ mice, the *Cyp1b1* expression level in the TCDD-exposed group was higher than that in the control group (Fig. 1). Although a slight decrease of *Ar* mRNA was seen in the $Ahr^{+/+}$ mice (TCDD exposed), we did not detect any significant change in *Ar* and *Srd5a2* mRNA levels among the three TCDD-exposed genotypes. *Pbsn* (dorsolateral), *Sbp* (ventral), and *Msmb* (lateral) were used to investigate functional cytodifferentiation levels of each prostatic epithelia, as reported by others [28]. These three prostate markers were expressed in the control UGCs on PND14. They were barely detectable in TCDD-exposed $Ahr^{+/+}$ and $Ahr^{+/-}$ mice, while TCDD-exposed $Ahr^{-/-}$ mice had quantities of these three marker mRNAs that were very similar to control mice (Fig. 1).

Histopathology of the Urogenital Complex

Prostatic lobes were found to be well developed in the control animals (Fig. 2, A and D). In the TCDD-exposed $Ahr^{+/+}$ animals, the prostatic lobes with existing epithelial layers were clearly observed (Fig. 2, B, C, E, and F). In situ hybridization analysis of the tissue section adjacent to the sections used for histopathological examinations revealed that epithelial cells of dorsolateral prostate lobes had *Pbsn* mRNA signals (Fig. 3A). In accordance with the RT-PCR data, no signals were detected in the epithelia of TCDD-exposed dorsolateral prostates (Fig. 3C).

Gene Expressions in Testis

Consistent with the analysis of UGC (Fig. 1), the expression of *Ahr* mRNA was not detected in the $Ahr^{-/-}$ testis, whereas the *Ahr* expression level was 2-fold higher in the $Ahr^{+/+}$ than in the $Ahr^{+/-}$ mice. No differences were detected in *Ar* mRNA levels (Fig. 4). Among the three CYP1 genes tested, *Cyp1a1* mRNA was not detected in any of the *Ahr* genotypes (data not shown). Although *Cyp1a2* and *Cyp1b1* mRNA was detected in the testis, there were no statistically significant differences among the three genotypes and between the control and TCDD-exposed testes (Fig. 4). The mRNA of *CLGN* and *Hspa2*, male germ cell-specific markers expressed in the pachytene stage of spermatocytes [29, 30], was observed at the same levels among the three genotypes, regardless of TCDD exposure (Fig. 4). No difference was observed in the expression level of *Shbg*, a protein secreted from Sertoli cells [31]. RNA expression levels of four steroidogenic enzyme genes for testosterone synthesis, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Hsd17b3*, were not affected in the three genotypes under the TCDD dosing regimen used (Fig. 4). Consistently, intratesticular testosterone levels in all genotypes and TCDD-exposed animals were not changed among the three genotypes, regardless of TCDD exposure (Table 1). Additionally the mRNA of *Akr1c4* and *Srd5a1* enzymes for synthesis of 5α-androstane-3α, 17β-diol, the major form of testicular androgen in immature mice [32], was not altered by TCDD exposure and showed no differences among the three genotypes in the testes (Fig. 4).

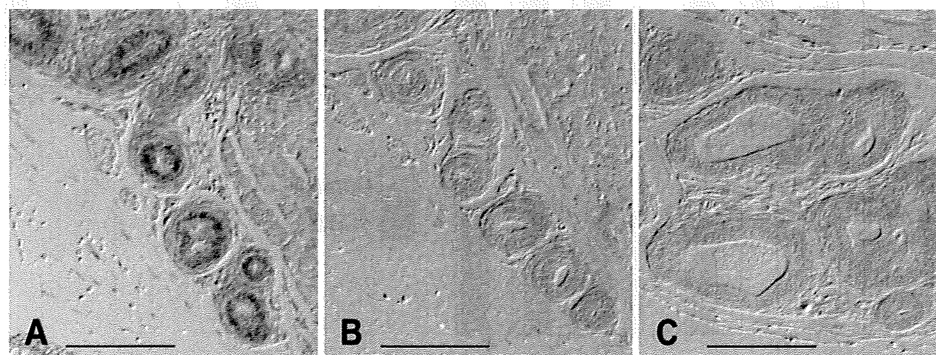

FIG. 3. In situ hybridization analysis of *Pbsn* mRNA expression in the dorsolateral prostate lobe of $Ahr^{+/+}$ mouse UGCs on PND14. **A**) Antisense probe for control mouse. **B**) Sense probe for control mouse. **C**) Antisense probe for TCDD-exposed mouse. Note that epithelial cells of the dorsolateral prostate lobes show *Pbsn* mRNA signals in control mice (**A**), but not in TCDD-exposed mice (**C**). Bar = 200 μm.

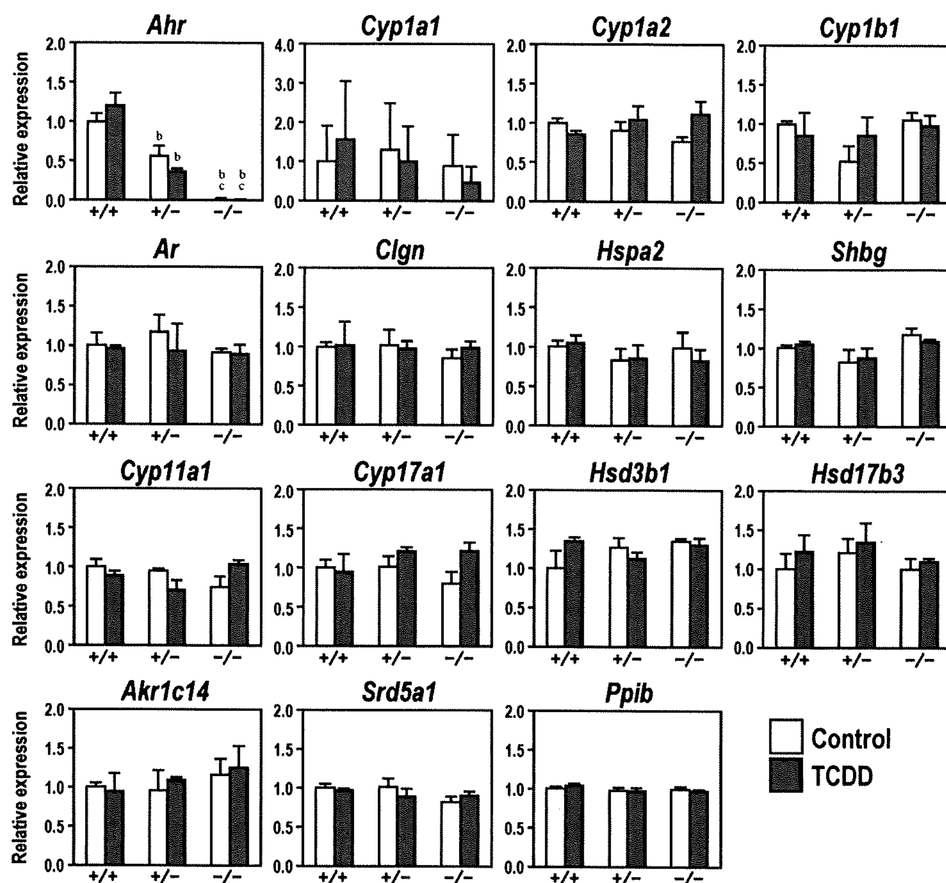

FIG. 4. Quantitative RT-PCR analysis of gene expression in the testes of male pups of three *Ahr* genotypes ($Ahr^{+/+}$, $Ahr^{+/-}$, and $Ahr^{-/-}$) on PND14 with or without TCDD exposure in utero. The values are expressed as the mean ± SE for three samples from each group. Significant differences were analyzed with ANOVA followed by the Fisher PLSD test (b, versus the same treatment of wild-type; c, versus the same treatment of heterozygous; $P < 0.01$).

Histopathology of the Testis

Spermatocytes at the pachytene stage proliferate from the spermatogonium on PND14, and calnexin-t is expressed at this stage [33]. In the present study, germ cells from the three mouse genotypes were immunostained for calnexin-t (Fig. 5, A–C). Positive cell populations were similar in control and TCDD-exposed testes from all genotypes (Fig. 5, D–F).

DISCUSSION

Ahr-Dependent Reduction in Anogenital Distance and Impairment of Prostatic Development by In Utero and Lactational TCDD Exposure

In this study, we demonstrated that in utero and lactational TCDD exposure caused reduction of the anogenital distance and impairment of prostatic development in an *Ahr*-dependent manner, because reduction of anogenital distance and disappearance of prostatic epithelial protein mRNAs were observed in $Ahr^{+/+}$ and $Ahr^{+/-}$ but not in the $Ahr^{-/-}$ offspring. The induction of *Cyp1a1* and *Cyp1b1* mRNAs was also observed in $Ahr^{+/+}$ and $Ahr^{+/-}$ mice but not in $Ahr^{-/-}$ mice. Taken together, impairment to the male reproductive system by in utero and lactational TCDD exposure was mainly dependent on the fetal *Ahr* gene.

A study using *Ahr* knockout mice that produced results similar to ours has been reported [17]. In that study, $Ahr^{+/-}$ female and $Ahr^{+/-}$ male mice were mated, and TCDD (5 μg/kg) was injected into the pregnant mice on GD13. The levels of prostatic protein markers were reduced in the TCDD-exposed $Ahr^{+/+}$ mice on PND90, but not in the $Ahr^{-/-}$ mice. Furthermore, those authors reported that TCDD administration on GD13 severely inhibited prostatic bud formation from the urogenital sinus in the fetus [28]. The use of in an vitro organ culture system as well as *Ahr* knockout mice revealed that the inhibition was mediated by AHR expressed in the mesenchy-

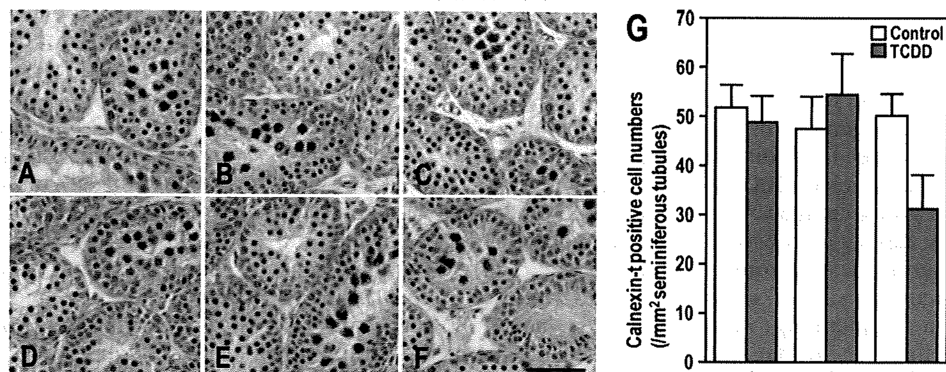

FIG. 5. Immunostaining of CLGN in the testes of male pups on PND14. Testis tissue preparations from each genotype (n = 3) were immunostained. **A**) Control $Ahr^{+/+}$ testis. **B**) Control $Ahr^{+/-}$ testis. **C**) Control $Ahr^{-/-}$ testis. **D**) TCDD-exposed $Ahr^{+/+}$ testis. **E**) TCDD-exposed $Ahr^{+/-}$ testis. **F**) TCDD-exposed $Ahr^{-/-}$ testis. CLGN-positive spermatocytes were observed in all genotypes and TCDD-treatment groups. **G**) Morphometric analysis. There were no statistical differences in the number of positive cells per testis cross section, for all genotypes and treatments. Bar = 100 μm.

mal cells of the urogenital sinus [34] and was not caused by interruption of androgen signaling in this tissue [35]. It is still unclear how TCDD impairs the responsiveness of the developing prostate to androgen.

In Utero and Lactational TCDD Exposure Decreased Androgen Responsiveness of the Prostate

In the histological examinations, the prostatic lobes developed with obvious epithelial layers in TCDD-exposed $Ahr^{+/+}$ mice, indicating TCDD-exposed prostates may function as normal exocrine glands (Fig. 2, B and C). However, using RT-PCR and in situ hybridization analyses, we could not detect prostatic epithelial secretory protein mRNAs in the TCDD-exposed UGCs (Figs. 1 and 3). Fewer layers of epithelial cells and increased cell numbers in mesenchymal cells were observed in the TCDD-exposed $Ahr^{+/+}$ animals, suggesting that in utero and lactational exposure to TCDD produced functional abnormalities (Fig. 2, E and F). The prostatic secretory proteins, PBSN, MSMB, and SBP, were reportedly upregulated via the androgen receptor [36–38]. Since no significant differences in intratesticular testosterone levels were found between control and TCDD-exposed animals on PND14, we speculated that abnormal development of prostate glands may be due to decreased androgen sensitivity or that TCDD disrupts mouse prostate epithelial cell differentiation into luminal epithelial cells. This notion is consistent with a previous study [9] in the sense that the ventral prostate of male rat offspring exposed to TCDD in utero and via lactation did not respond to the exogenous androgens testosterone, 5α-dihydrotestosterone (DHT), and 5α-androstane-3α, 17β-diol, in the organ culture system. Administration of the androgen receptor antagonist flutamide and the 5α-reductase inhibitor finasteride to rats in the late pregnancy period did not cause prostate growth in male offspring on PND60 [39], suggesting that DHT is an essential steroid hormone for prostate development. Since the inhibition was found specifically in the prostate but not in the seminal vesicle, the reduction in DHT production in the prostate was initially hypothesized to occur in males exposed to TCDD in utero and via lactation. However, 5α-reductase type 2 enzymatic activity and mRNA expression was elevated, compared to control groups [9, 10]. Thus, it is reasonable to speculate that decreased androgen responsiveness in the TCDD-exposed offspring was caused by other factors. Our previous study using Holtzman rats showed significantly reduced androgen receptor mRNA expression in the ventral prostate following in utero and lactational TCDD exposure, suggesting that the decreased androgen responsiveness might be due to reduced amounts of receptor molecules [10]. However, we could not detect a significant reduction in androgen receptor mRNA here, probably due to a difference in animal species or organs used for RT-PCR analysis.

Function of AHR in the Testis

AHR was reportedly responsible for apoptotic signaling, and the number of primordial cells in the ovarian germ line was not attenuated due to a defect in the apoptosis in *Ahr*-null female mice [20]. More recently, testicular dysfunction was reported in aged *Ahr*-null mice [40], and HSD3B1 expression in Leydig cells was significantly reduced at 24 wk, resulting in serum testosterone decline, lowered sperm number, and reduced size of seminal vesicles. AHR seems to play a role in maintaining normal steroidogenesis in aged animals. However, in that study, there were no significant differences in testicular functions between wild-type and *Ahr*-null mice during younger stages (10 wk old). In our present study, we were unable to find differences in testicular functions among the *Ahr* genotypes, including testosterone production, Sertoli cell differentiation, and spermatogenic cell differentiation. Therefore, it is reasonable to conclude that AHR has very little function in early stages of gonad development. If the AHR functions even at the early stage of development, the functional redundancy with other genes may also exist among *Ahr* and other genes during testicular development.

Resistance of Testicular Development to In Utero and Lactational TCDD Exposure

In our previous study, TCDD administration to pregnant Holtzman rats on GD15 did not alter the testicular weight or serum testosterone levels of male offspring on PND49 [10]. In our present study, we did not find any differences in testicular cell differentiation levels between control *Ahr*-carrying animals and TCDD-exposed animals that survived the high dose of TCDD exposure, including supporting cell marker and spermatogenic cell differentiation markers, which suggested that even in the TCDD-exposed animals testicular differentiation proceeded normally. 5α-androstane-3α, 17β-diol, the major form of testicular androgen in immature mice [32], was previously reported to be slightly reduced in PND21 mice perinatally exposed to TCDD [17]. However, in our present study, in the assayed testes from PND14, no difference was seen in both *Akr1c4* and *Srd5a1* mRNA expression levels among the three genotypes and TCDD treatment. Moreover, in utero and lactational exposure to TCDD did not alter the expression levels of steroidogenic enzyme genes in Leydig cells in $Ahr^{+/+}$ and $Ahr^{+/-}$ animals. At a relatively high dose, testicular CYP11A1 activity was reduced by TCDD exposure [41]. In our previous report, we also found that administration of 100 μg TCDD/kg to adult male mice reduced testicular *Cyp11a1* mRNA and protein levels [42], and in vitro co-planer PCB (3,3′,4,4′,5-pentachlorobiphenyl; PCB126) exposure to neonatal mouse testis downregulated *Cyp11a1* mRNA expression [33]. The reason why we could not detect the reduction in *Cyp11a1* mRNA in the present study is not clear, but it is speculated that intratesticular levels of TCDD in male pups born to dams given TCDD on GD13 was not sufficient to downregulate *Cyp11a1* by PND14.

Although testes and UGCs were collected from the same individual pups, both *Cyp1a2* and *Cyp1b1* mRNAs were not induced in the testes of mice with any of the three genotypes, whereas in the UGCs, *Cyp1a2* and *Cyp1b1* were significantly induced. Moreover, an approximately 30-fold increase in *Cyp1a1* was observed in the $Ahr^{+/+}$ UGCs. Thus, UGCs are much more sensitive to TCDD than testis in terms of *Cyp1a1* mRNA induction. Using a xenobiotic-responsive element connected to the β-galactocidase reporter gene, a transgenic mouse line was generated and then exposed to TCDD in utero and via lactation [43]. X-Gal staining analysis clearly demonstrated that fetal urogenital tracts showed significant induction of the reporter gene, but that the testis did not respond. Based on the above-mentioned results, we speculated that UGCs express modulating factors (modifiers) that enhance AHR-mediated transcription, and that these were lacking or present in small amounts in the testis. Although it cannot be excluded that tissue concentration of TCDD in the testis was lower than that in the UGC, it is more likely that the testis is much more resistant to TCDD exposure than the UGC.

In conclusion, using *Ahr* knockout mice, we confirmed that in utero exposure to TCDD caused *Ahr*-dependent impairment of prostate development and reduced anogenital distances in

male offspring, but that the testicular development seemed to be resistant to TCDD exposure. AHR was not associated with testicular development under physiological conditions.

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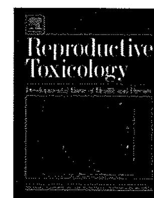

When does the sex ratio of offspring of the paternal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure decrease: In the spermatozoa stage or at fertilization?

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ABSTRACT

Recent animal experiments confirmed that paternal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure decreases the sex ratio of offspring at birth without altering litter size. However, the timing of this decrease remained unclear. Male mice were administered TCDD at 7–12 weeks of age and mated with non-treated females. The Y-bearing/X-bearing sperm ratio was examined by real-time PCR and FISH methods, and the sex ratio of the 2-cell embryos collected from non-treated females that had been mated with TCDD-exposed males were investigated by nested PCR. The Y-bearing/X-bearing sperm ratio was not significantly decreased in the TCDD group. However, the sex ratio of the 2-cell embryos of the TCDD group was significantly lower than that of the control group. These results may have resulted from a decrease in fertility of Y-bearing sperm. Thus, the results of this study suggested that the sex ratio of the offspring was decreased at fertilization and not during the spermatozoa stage.

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1. Introduction

In recent years the public has become more aware that exposure of males to certain environmental or occupational agents affects their offspring. Occupational exposure in various industries has led to increased incidences of miscarriage [1] and various birth defects [2]. It has also been shown that paternal cranial irradiation leads to epigenetic alterations in offspring [3]. Prominent among these reports are those by Mocarelli et al. [4,5] showing that paternal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in Seveso, Italy, led to a decrease in male offspring.

In 1976, TCDD was released in an explosion at a chemical plant near Seveso, Italy, resulting in the highest concentrations of TCDD ever recorded in humans. Subsequent data linked a decrease in male births in Seveso to the increased TCDD concentration in fathers, and the altered sex ratio (the proportion of male offspring) was especially pronounced in the children of fathers exposed to TCDD before age 19 [4,5]. In addition to the Seveso incident, Ryan et al. [6], in a study conducted in Ufa, Russia, suggested that human exposure to high levels of dioxin is associated with the birth of more girls only in cases of paternal exposure. In our previous study, we exposed young male mice to two concentrations of TCDD (TCDD2/0.4 group and TCDD2000/400 group; an initial loading dose of 2 or 2000 ng TCDD/kg, followed by a weekly maintenance dose of 0.4 or 400 ng TCDD/kg) to re-create the Seveso incident and evaluated the sex ratio of their offspring at birth [7]. The reason why we used young male mice (7 weeks at the start of administration) in that study was that the sex ratio of offspring of males exposed to TCDD during adolescence showed a greater decrease than that of males who were older than that when the incident occurred [4,5]. The results from the previous study revealed that paternal TCDD administration produced a dose-dependent reduction in the sex ratio of offspring (F1) and a significantly lower proportion of male offspring in the high-dose (TCDD2000/400) group. In addition, the induction intensity of CYP1A1 in the liver varied among individuals in the TCDD group, and the dimensions of the CYP1A1 immunoreactive area were correlated with the sex ratio of the offspring. This means that the high sensitivity subgroup of male parents to TCDD was strongly related to the decrease in male offspring. We also reported that TCDD exposure does not influence

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litter size; the number of male offspring decreases by TCDD exposure, while the number of female offspring increases. From these data, we presumed that paternal TCDD exposure decreased the sex ratio of offspring and altered it before implantation occurred. However, the mechanisms underlying the reduction in male offspring and the timing of this change have remained unclear.

The purpose of this study was to investigate when the sex ratio of the offspring decreases. We examined the Y-bearing/X-bearing sperm ratio as well as the sex ratio of 2-cell embryos by exposure to the same dose (an initial loading dose of 2000 ng TCDD/kg, followed by a weekly maintenance dose of 400 ng TCDD/kg) as used in our previous study [7], because this dose group showed a significant decrease in the sex ratio of offspring at birth. The effects of TCDD exposure to sperm concentration and motility were also examined.

2. Materials and methods

2.1. Chemicals

TCDD was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sesame oil, used for dissolving TCDD and for vehicle treatment as a control, was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Animals and treatments

Male and female ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and bred at Kobe University (Kobe, Japan). They were maintained under controlled conditions of temperature (23 ± 2 °C) and humidity ($50 \pm 10\%$) on a 12 h light, 12 h dark cycle. The animals were given an MR-A1 laboratory diet (Nosan Corporation, Yokohama, Japan) and filtered water *ad libitum* throughout the experiments. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 19-5-46) and carried out according to the Kobe University Animal Experimentation Regulations.

We used 7-week-old male mice at the start of administration to re-create the Seveso incident. These male mice were divided into a control group ($n = 59$) and a TCDD group ($n = 49$), and were administered TCDD orally by gastric sonde with an initial loading dose of 2000 ng TCDD/kg body weight or an equivalent volume of sesame oil (vehicle) as a control, followed by a weekly maintenance dose of 400 ng TCDD/kg body weight to maintain the body TCDD burden as constantly as possible or an equivalent volume of sesame oil until the mice reached 12 weeks of age. We also used 6-week-old female mice ($n = 62$) for mating with males.

2.3. Epididymal sperm concentration and motility analysis

Mouse spermatozoa were collected from TCDD-treated mice and control mice for examination of sperm concentration and sperm motility. The mice were sacrificed by cervical dislocation under anesthesia using ether, and the caudal epididymides were then dissected out. The epididymis was cut at one or two points with a scissors, and white sperm pellets were released into a TYH-HEPES medium droplet that was covered with mineral oil (Nacalai Tesque Inc., Kyoto, Japan) on a 37.5 °C heater plate. The TYH-HEPES medium was composed of 119.37 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 1.00 mM sodium pyruvate, 5.56 mM glucose, 25.07 mM HEPES, 0.05 g/l streptomycin sulfate, 100 U/ml potassium penicillin G, 0.1% polyvinylalcohol (Sigma–Aldrich Co., St. Louis, MO, USA) and 5 mg/l phenol red. The recovered spermatozoa were diffused for 5 min in the droplet. The sperm concentration was measured using a hemocytometer chamber. For sperm motility analysis, a 2 μl drop of sperm suspension was put on a 1-mm-deep stage of a glass plate to assess sperm motility (Fujihira Industry Co., Ltd., Tokyo, Japan), covered with the coverslip and then placed on the heated plate (37.5 °C) under a bright-field microscope (EX41; Olympus Co., Tokyo, Japan). Sperm motility was recorded with a CCD camera (CS230B; Olympus Co.) and a DVD recorder (DVR-7000; Pioneer Co., Tokyo, Japan). The motility patterns of more than 100 spermatozoa (except large sperm clumps) were randomly recorded with the CCD camera and DVD recorder in each sample. The recorded movies were played in the slowest mode. At least 10 microscopic fields were observed for each sample, and the percentage of motile sperm was determined.

2.4. Sperm DNA isolation

Mouse spermatozoa for DNA isolation were collected from TCDD-treated and control mice by pricking the caudal epididymides with needles, and this manipulation was done in a TYH-HEPES medium droplet that was covered with mineral oil under a stereomicroscope so as not to contaminate the other male tissues. The sperm pellets were resuspended with a 1–1.5 volume of lysis buffer containing 0.5 M EDTA, 2-mercaptoethanol (Sigma–Aldrich Co.) and 10 mg/ml proteinase K (Takara Bio Inc., Shiga, Japan), and then incubated in a shaking water bath at 55 °C overnight. The 2-mercaptoethanol was used to obtain high-quality spermatozoa DNA yields for real-time PCR, because the spermatozoa DNA is tightly packed into protamines. Sperm DNA extraction was performed using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

2.5. Real-time PCR and calculation of Y-bearing/X-bearing sperm ratio

To gain an accurate prediction of the X and Y chromosome content in the sperm DNA samples, quantitative real-time polymerase chain reaction (PCR) analysis was performed using a LightCycler (Roche Co., Basel, Switzerland). We selected the *Sry* primer pair (forward: 5′-ATGGAGGGCCATGTCAAGCG-3′ and reverse: 5′-GGGTATTTCTCTCTGTGTAGGATCTTCAA-3′) as Y-bearing sperm-specific primers and the *AR* primer pair (forward: 5′-ATGGAGGTGCAGTTAGGGCT-3′ and reverse: 5′-TCCTCAGTGTGCTGCTGCC-3′) as X-bearing sperm-specific primers. To calculate Sry and AR gene copy numbers in the isolated genomic DNA, the basic protocol for real-time PCR was briefly modified [8]. The Sry and AR gene PCR products were used for calibration by calculating the molecular weight and making stock dilutions from 2×10^8 to 2×10^3 copies/μl. Aliquots (2 μl) of DNAs or standard DNA solution were amplified with a master mixture (SYBR Premix Ex Taq (Perfect Real Time), TaKaRa Bio Inc.) containing the Sry- or AR-specific primers described above in a final volume of 20 μl. Fluorescent products were detected at the end of the extension period. The specificity of the amplified PCR products was confirmed by melting curve analysis. Unknown concentrations of samples were extrapolated by a comparison with standards amplified under the same conditions using Lightcycler software. The Y-bearing/X-bearing sperm ratio [DNA concentration of Sry/DNA concentration of AR] of each animal was calculated.

2.6. Preparation of epididymal sperm smears for FISH

Mouse spermatozoa for FISH analyses were collected from TCDD-treated and control mice. Mice were killed by cervical dislocation, and then the caudal epididymides were dissected out. The epididymis was cut at 1 or 2 points with a scissors, and the white sperm pellet was placed into 0.1 ml of 2.2% sodium citrate at 32 °C for 5 min to allow the sperm to swim out into the solution. The sperm suspension was centrifuged at 1500 rpm for 5 min. The sperm pellet was resuspended into 0.1 ml of 0.075 M KCl for 45 min at 37 °C. Sperm suspension (5 μl) was pipetted onto a dry glass slide that had been precleaned by soaking in 100% ethanol for at least 24 h. The smears were allowed to air-dry overnight and then stored at −20 °C until used.

2.7. FISH in spermatozoa and calculation of Y-bearing sperm ratio

Smears of mice were each fixed in 3:1 methanol: acetic acid and air dried before pretreatment commenced. To prepare the smears for FISH, spermatozoa were decondensed by incubating the slides for 30 min in 0.2 ml of 10 mM dithiothreitol (Sigma–Aldrich Co.) on ice. Slides were briefly rinsed in D.W. and allowed to dry completely at room temperature before they were used for hybridization. The probes specific for mouse Y chromosome (Cy3-labeled) were obtained from Cambio (Cambridge, UK). The probe mixture, which included labeled probes and hybridization buffer, was denatured at 72 °C for 10 min. The sperm smears were denatured at 78 °C for 6 min in 70% formamide/2xSSC and then dehydrated in an alcohol series (70%, 85% and 100%, 2 min each). The probe mixture was applied to the sperm smears and incubated at 37 °C overnight. After hybridization, the slides were washed six times: three times in washing solution (50% formamide/2xSSC), twice in 2xSSC, and once in PN buffer for 5 min each at 37 °C. The nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) (Cambio Ltd.). Coverslips were applied and sealed with clear nail polish. Slides were then viewed.

The slides were examined with a Leica FW4000 fluorescence microscope (Leica Co., Wetzlar, Germany) (magnification: 400×) equipped with single and double band-pass filters to detect DAPI and Cy3. The investigation was performed blindly, and at least 1000 cells per slide were scored as much as possible by assessing randomly selected visual fields. Sperm nuclei were scored only when they were morphologically preserved, not clumped or overlapping, when they showed well-defined outlines and when the sperm heads were not decondensed to more than twice the size of normal non-decondensed spermatozoa. In every sample, the proportion of sperm presenting with a clear signal was ≥95%.

The Y-bearing sperm ratio [Y-chromosome-bearing spermatozoa/DAPI-positive spermatozoa × 100] of each animal was calculated.

2.8. Embryo collection for nested PCR

Six-week-old female mice were superovulated by intraperitoneal injections of 5 IU eCG (Teikoku Hormone Medical Co., Ltd., Tokyo, Japan) followed 48 h later by intraperitoneal injections of 5 IU of hCG (Teikoku Hormone Medical Co., Ltd.). One week after the last exposure of TCDD to male mice, each superovulated female mouse that had not been exposed to TCDD was paired with a male, with one pair per cage. After confirmation of vaginal plug in each female, embryos were collected 44 h after hCG injection from the oviducts of the females at the 2-cell stage in 37 °C FHM medium, which was composed of 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 4 mM $NaHCO_3$, 0.2 mM glucose, 0.2 mM glutamine, 0.2 mM Na-pyruvate, 10 mM Na-lactate, 1.71 mM $CaCl_2 \cdot 2H_2O$, 0.01 mM EDTA·4Na, 20 mM HEPES, 80 mg/l kanamycin and 1 g/l BSA.

The embryos were transferred to 80 μl drops of KSOM medium, which was composed of 94.97 mM NaCl, 2.55 mM KCl, 0.37 mM KH_2PO_4, 0.23 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 0.22 mM glucose, 1.0 mM glutamine, 0.2 mM Na-pyruvate, 10 mM Na-lactate, 1.7 mM $CaCl_2{\cdot}H_2O$, 80 mg/l kanamycin, 3 g/l BSA, 0.1 mM EDTA-2Na under mineral oil and separated by gentle pipetting of the groups of 2-cell embryos using a micropipette with an internal diameter of 70–100 μm. Each 2-cell embryo was put into a PCR micro-test-tube containing 15 μl of 0.15 mg/ml proteinase K with the help of a dissection microscope. After the embryos were collected, the PCR tubes were heated for 60 min at 50 °C and then for 10 min at 100 °C to inactivate the proteinase K. The cells in the PCR micro-test-tubes were stored at −80 °C until PCR analysis was performed.

2.9. Oligonucleotide primers for nested PCR

The *Sry* gene was chosen as the Y chromosome-specific gene and the *IL-3* gene was chosen to detect both XX and XY embryos. For every locus, two sets of primers were used, an inner and an outer set. The primers were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The oligonucleotide sequences from the outer primers used in the initial PCR were as follows:

Sry-outer forward 5′-TCTTAAACTCTGAAGAAGAGAC-3′
Sry-outer reverse 5′-GTCTTGCCTGTATGTGATGG-3′
IL-3-outer forward 5′-GGGACTCCAAGCTTCAATCA-3′
IL-3-outer reverse 5′-TGGAGGAGGAAGAAAAGCAA-3′
The inner primer sequences used in the second-round PCR were as follows:
Sry-inner forward 5′-TTCCAGGAGGCACAGAGATT-3′
Sry-inner reverse 5′-GTCCCACTGCAGAAGGTTGT-3′
IL-3-inner forward 5′-GGGAAGCTCCCAGTGAGTAA-3′
IL-3-inner reverse 5′-GGTTCACCCACAGCTGTCTT-3′

The sequences of Sry-outer primers were the same as those used in Kunieda et al. [9].

2.10. Nested PCR and the sex ratio of the 2-cell embryos

A multiplex PCR reaction using nested primers was performed in two rounds for simultaneous amplification of the *Sry* and *IL-3* sequences. Samples were thawed on ice, and a 15 μl first-round PCR mix containing the outer primers was added. After the first amplification, 1 μl portions of the products of the amplification reactions were dispensed respectively into each tube, and each was subjected to second-step amplification with 9 μl of a reaction mixture containing the corresponding inner primers. Both steps of the PCR were carried out using a reaction mixture consisting of a 5× PCR buffer, 2.5 mM dNTPs, 50 μM each of the oligonucleotide primers and 1.25 U/μl of Taq DNA polymerase (Promega). PCR was performed for 30 cycles, each consisting of 1 min denaturation at 94 °C followed by annealing and extension for 1 and 1.5 min each at 55 and 72 °C, respectively. The PCR products were subjected to electrophoresis in a 2.5% agarose gel containing 0.005% ethidium bromide. The male embryos showed both Sry and IL-3 signals, but the female embryos showed only the IL-3 signal.

The sex ratio of the 2-cell embryos [number of male embryos/number of (male + female) embryos × 100] of each animal was calculated.

2.11. Statistical analysis

SAS Ver 5.0 (SAS Institute, Cary, NC, USA) was used to analyze the present data. In comparing every parameter between two groups (control and TCDD groups), the equality of variances was evaluated by using the *F* test to select statistical tests. If variances were homoscedastic, we used Student's *t*-test. In contrast, if the variances of parameters were not homoscedastic, the Welch test was applied. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Epididymal sperm concentration and motility

Epididymal sperm concentrations of five males per group and sperm motility of seven males in the control group and five males in the TCDD group were examined. Figs. 1 and 2 show the effects of TCDD exposure on sperm concentration and motility. Compared with the control group, the TCDD group showed a lower level of caudal epididymal sperm concentration [Control: 4.28 ± 0.79 × 10^7/ml; TCDD: 2.29 ± 0.70 × 10^7/ml; P = 0.10], because one of the mice in the TCDD group showed a very low sperm concentration. The mean value of sperm motility in the TCDD group was lower than that of the control group, but the difference was not significant [Control: 45.39 ± 7.19%; TCDD: 42.48 ± 5.71%; P = 0.77].

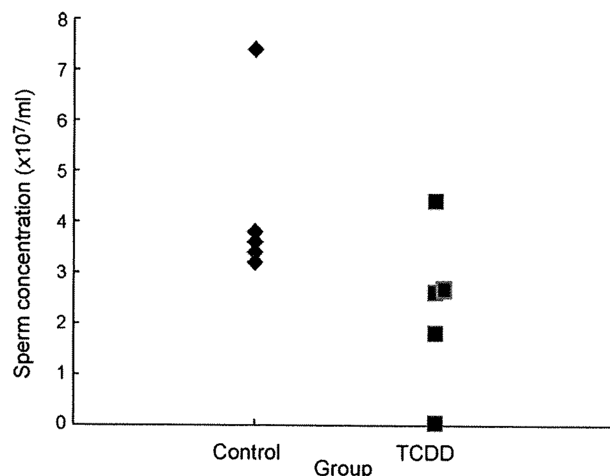

Fig. 1. Concentration of epididymal spermatozoa. The sperm concentration of the TCDD group was lower than that of the control group (Control: 4.28 ± 0.79 × 10^7/ml; TCDD: 2.29 ± 0.70 × 10^7/ml; P = 0.10). The sample size was five per group (n = 5).

3.2. Y-bearing/X-bearing sperm ratio

The results of quantitative PCR are shown in Fig. 3A–C. The control group consisted of 20 male mice and the TCDD group consisted of 14 male mice. Although the Y-bearing/X-bearing sperm ratio of the TCDD group was lower than that of the control group [Control: 2.68 ± 0.15, TCDD: 2.36 ± 0.04; P = 0.060], the difference was not significant. In addition, the TCDD group tended to have lower levels of Sry DNA concentrations [Control: 28.12 ± 1.20 ng/μl, TCDD: 25.80 ± 0.61 ng/μl (P = 0.096)], which is a sensitive and specific marker of the Y chromosome. This difference also did not attain statistical significance. The DNA concentrations of AR showed no differences between the control and TCDD groups [Control: 10.87 ± 0.57 ng/μl, TCDD: 10.95 ± 0.29 ng/μl, P = 0.895].

3.3. Y-bearing sperm ratio examined by FISH method

A total of 10,083 spermatozoa from five males per group were scored with the FISH technique (Fig. 4). As shown in Table 1, no significant differences were found between the groups. The sperm

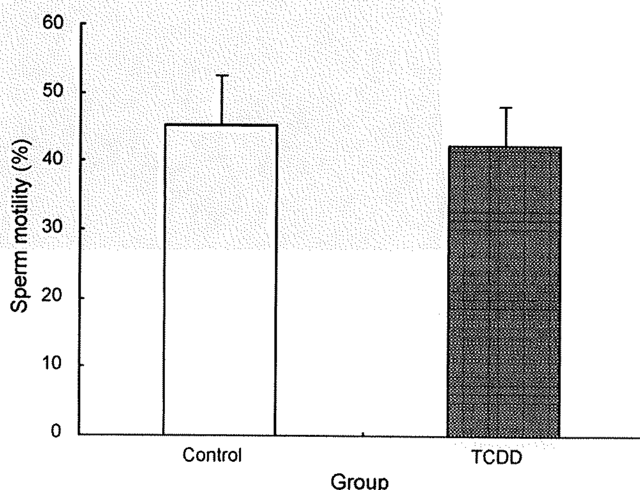

Fig. 2. Motility of epididymal spermatozoa. There were no apparent changes in the motility of the cauda epididymal spermatozoa in the TCDD group compared with the control group (P = 0.77). However, the mean value in the TCDD group was lower than that in the control group (Control: 45.39 ± 7.19%; TCDD: 42.48 ± 5.71%). The sample size of the control group was seven, and that of the TCDD group was five. Each histogram and bar indicate mean ± SE.

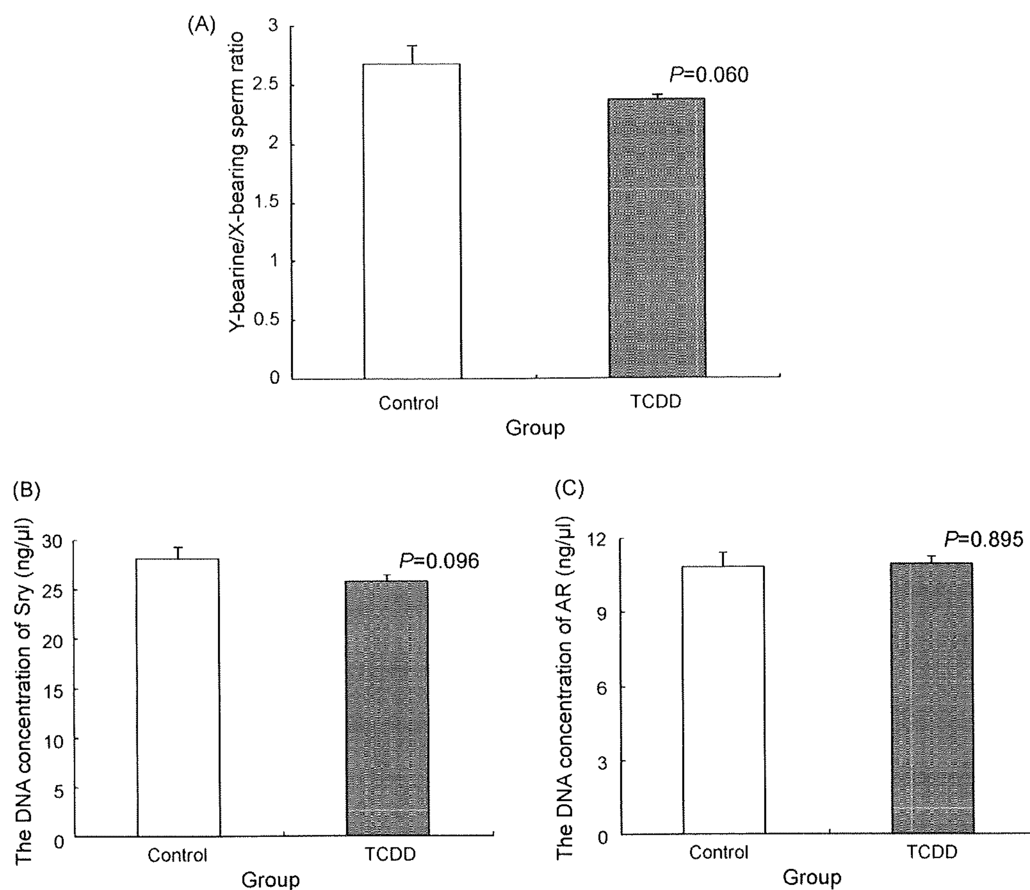

Fig. 3. (A) Y-bearing/X-bearing sperm ratio. The Y-bearing/X-bearing sperm ratio in the TCDD group did not show a significant decrease compared with the control group. However, the Y-bearing/X-bearing sperm ratio of the TCDD group was lower than that of the control group. The sample size of the control group was 20, and that of the TCDD group was 14. (B) The concentrations of the sperm DNA of Sry. The Sry primer was used to detect Y-bearing sperm. The concentrations of sperm DNA of Sry in the TCDD group were lower levels than those of the control group. (C) The concentrations of the sperm DNA of AR. The AR primer was used to detect X-bearing sperm. The concentrations of AR showed no apparent changes between the control group and the TCDD group. Each histogram and bar indicate mean ± SE.

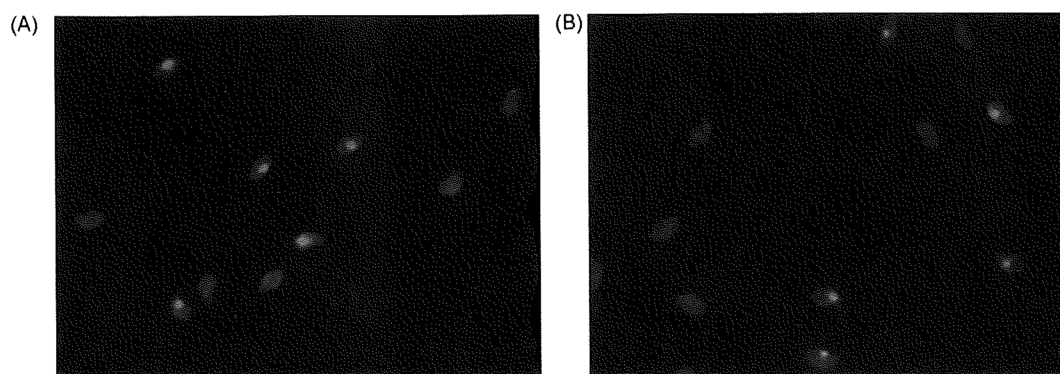

Fig. 4. Microscope images of FISH in epididymal spermatozoa. Samples were hybridized with chromosome-specific painting DNA probes for Y chromosomes (Cy3/red). The sperm nuclei were stained with DAPI and appear as blue. (A) Sperm samples of the control group yielded nearly equal ratios of X and Y chromosome-bearing cells. (B) Microscope images of FISH in epididymal spermatozoa of the TCDD group. There was no significant change in the Y-bearing sperm ratio of the TCDD group compared with the control group. The sperm nuclei were stained with DAPI.

Table 1
Y-bearing sperm ratio examined by FISH method.

Group	Control						TCDD					
Animal no.	1	2	3	4	5	Total	1	2	3	4	5	Total
Cells scored	1055	1071	1080	1086	1068	5360	1074	1079	1094	308	1168	4723
Y sperm	532	539	541	553	536	2701	539	540	549	149	586	2363
X sperm	523	532	539	533	532	2659	535	539	545	159	582	2360
Y-bearing sperm ratio (%)	50.43	50.33	50.09	50.92	50.19	50.39	50.19	50.05	50.18	48.38	50.17	50.03

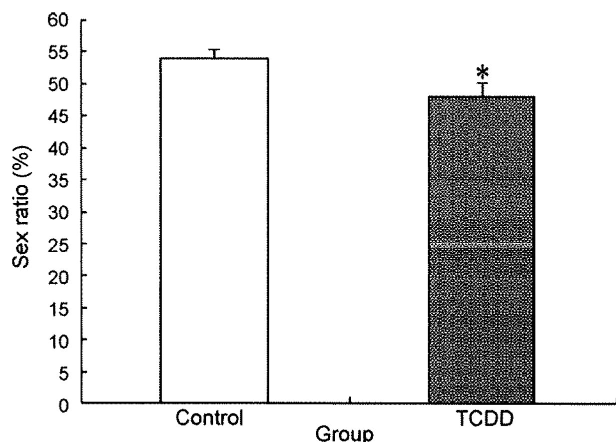

Fig. 5. Sex ratio of 2-cell embryos. A total of 614 embryos of the control group and 646 embryos of the TCDD group were examined. The proportion of XY 2-cell embryos from the TCDD-exposed sires was significantly lower than that of the control group. Each histogram and bar indicate mean ± SE. *: $P < 0.05$.

count scored with the FISH technique from one of the five mice in the TCDD group was very low, because the total number of sperm collected from the cauda epididymides of that mouse was much lower than that of any of the others.

3.4. Sex ratio of 2-cell embryos

The sex ratio of the 32 litters from the control group (614 embryos) and 30 litters from the TCDD group (646 embryos) were sexed by nested PCR using XY embryo-specific primers (Fig. 5). We found a statistically significant decrease in the sex ratio of embryos in the TCDD group compared with the control group [Control: 53.95 ± 1.54%, TCDD: 47.92 ± 2.20%, $P < 0.05$].

4. Discussion

In theory, the sex allocation ratio of many species of mammals is almost 1:1. However, in reality this is not necessarily true. There have been many reports about changes in the sex ratio of human offspring. Severe periconceptional life events have been found to reduce the sex ratio in offspring [10]. The proportion of male offspring decreases with increasing parental age, and is higher in white people than in black people [11]. The sex ratio varies with the coital rate and with the time taken to achieve conception [12]. The proportion of male offspring to female offspring decreased after the Kobe earthquake [13]. Among these reports about alterations in the sex ratio of offspring, reports that TCDD exposure altered the ratio have attracted attention. A decreased male/female sex ratio among children born to males exposed to TCDD at a relatively young age compared with unexposed males has been reported in Seveso, Italy [4,5] and Ufa, Russia [6]. Still other reports found no significant association between paternal serum TCDD levels and the sex ratio of offspring in the USA [14] and Japan [15]. In animal experiments, Ikeda et al. [16] showed that TCDD exposure to male rats *in utero* significantly decreased the number of male offspring. In contrast, Rowlands et al. [17] showed that the number of rat male offspring was not decreased by *in utero* TCDD exposure. Thus, based on these previous articles, it was unclear whether or not TCDD exposure altered the sex ratio of offspring, and no experiments re-creating the Seveso incident had been conducted. Therefore, in our previous study we exposed two different doses of TCDD only to sexually mature young male mice (F0) and examined TCDD's effect on the sex ratio of their offspring (F1) at birth [7]. The results showed that the male/female sex ratio of the offspring dose-dependently decreased in the TCDD groups, and that of the high-dose group significantly decreased. Despite changes in the sex ratio, no alteration was found in the litter size of the TCDD group compared to the control group [7]. The data suggest that direct paternal TCDD exposure, not *in utero* exposure, decreases the sex ratio of offspring, and that the sex ratio of offspring already changes before implantation signs are seen. However, the timing of the alteration of the sex ratio of offspring in TCDD-exposed male parents remained unclear, so we investigated TCDD exposure's effect on the Y-bearing/X-bearing sperm ratio and the sex ratio of 2-cell embryos.

In the present study, the Y-bearing/X-bearing sperm ratio was examined by using a modified version of Parati's method [18] of quantitative PCR. This ratio did not show a significant decrease between the control group and the TCDD group. However, the Y-bearing/X-bearing sperm ratio and DNA concentrations of Sry of the TCDD group tended to decrease, with $P = 0.06$. On the other hand, the DNA concentration of AR was not affected by TCDD exposure. The Y-bearing sperm ratio was also checked by the FISH method, which revealed no marked differences between the groups. This result supports the findings of real-time PCR.

In this study, sperm motility was not significantly decreased by TCDD administration, but the mean value of that of the TCDD group was lower than that of the control group. In addition, the sperm concentration of the TCDD group was lower than those of the control group, with $P = 0.10$. Mocarelli et al. [19] reported that exposure to TCDD in infancy significantly reduced sperm concentration and motility, whereas an opposite effect was seen with exposure during puberty in Seveso, Italy. However, in the present study, the sperm concentration and motility were not significantly decreased. Our results did not coincide with those of a report on humans [19]. The causes of difference between ours and humans are that the parameters from sperm samples collected 22 years after the accident were measured in the human study, whereas we exposed TCDD to male mice from 7 to 12 weeks of age after puberty and examined their sperm almost immediately (1 week) after exposure. In addition, our TCDD administration dose was much lower than that in the Seveso incident. Thus, the differences in when the sperm samples were collected and in the exposed dose between Mocarelli's study [19] and the present one might account for the lack of a significant decreases in sperm concentration and motility in the present study.

The sex ratio of the 2-cell embryos in the TCDD group showed a significant decrease compared with the control group. This indicates that the sex ratio of the TCDD group had already decreased at the 2-cell embryo stage.

Thus, TCDD exposure significantly reduced the sex ratio of the embryos and the sex ratio at birth without altering litter size. However, TCDD exposure did not affect the Y-bearing/X-bearing sperm ratio. These results suggest that the sex ratio of offspring was decreased at fertilization, and thus that the sex ratio of neonates was also decreased. In the case of paternal TCDD exposure, the number of eggs per litter was not affected, therefore we think this is because the fertility of Y-bearing sperm might have been affected by TCDD exposure, and thus more X-bearing spermatozoa than Y-bearing spermatozoa were fertilized. As a result, both the sex ratio of embryos and that at birth were decreased in the TCDD group with no change in litter size. This distortion of the sex ratio might be produced by epigenetic alterations induced by TCDD exposure [20,21]. However, the mechanisms of decrease in the sex ratio, especially in the fertility of Y-bearing spermatozoa, remain to be investigated.

In conclusion, this study suggests that TCDD exposure to only male mice significantly decreases the sex ratio of 2-cell embryos without altering the Y-bearing/X-bearing sperm ratio. Our previous study revealed that the sex ratio at birth was significantly decreased by TCDD exposure despite no difference in litter size [7]. That finding, together with the current results, leads us to conclude that the sex ratio of offspring was decreased at fertilization in the TCDD exposure cases. Further investigation of the

differences in fertility between Y-bearing and X-bearing spermatozoa is needed.

Conflicts of interest

The authors have no conflicts of interest that would have inappropriately influenced the work presented in this manuscript.

Acknowledgments

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REPRODUCTION
RESEARCH

Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: a possible estrogenic effect of di(*n*-butyl) phthalate

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Abstract

Although di(*n*-butyl) phthalate (DBP), a suspected endocrine disruptor, induces testicular atrophy in prepubertal male rats, whether it exerts estrogenic activity *in vivo* remains a matter of debate. In the present study, we explored the estrogenic potency of DBP using 3-week-old male rats, and then examined the relationship between estrogen-induced spermatogenic cell apoptosis and testicular steroidogenesis. Daily exposure to DBP for 7 days caused testicular atrophy due to loss of spermatogenic cells, whereas testicular steroidogenesis was almost the same with the control values. A single exposure of DBP decreased testicular steroidogenesis in addition to decreasing the level of serum LH at 3 h after DBP treatment, with an extremely high incidence of apoptotic spermatogenic cells at 6 h after administration. To elucidate the estrogenic activity of DBP, we carried out an inhibition study using pure antiestrogen ICI 182,780 (ICI) in a model of spermatogenic cell apoptosis induced by DBP or estradial-3-benzoate (EB). Although both the DBP- and EB-treated groups showed a significant increase in spermatogenic cell apoptosis, ICI pretreatment significantly decreased the number of apoptotic spermatogenic cells in these two groups. In contrast, testicular steroidogenesis and serum FSH were significantly reduced in all the treated groups, even in the DBP+ICI and EB+ICI groups. Taken together, these findings led us to conclude that estrogenic compounds such as DBP and EB induce spermatogenic cell apoptosis in prepubertal rats, probably by activating estrogen receptors in testis, and that reduction in testicular steroidogenic function induced by estrogenic compounds is not associated with spermatogenic cell apoptosis.
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Introduction

Di(*n*-butyl) phthalate (DBP) is one of the most widely studied phthalate esters that disrupt the growth of normal reproductive organs, because of its wide use as a plasticizer in cosmetics, printing inks, and pharmaceutical coatings. The most prominent effect of DBP is testicular atrophy (Oishi & Hiraga 1980, Gray *et al.* 1982). To date, several mechanisms have been proposed to explain the induction of testicular atrophy by DBP, such as the depletion of zinc (Oishi & Hiraga 1980), increased oxidative damage of proteins, lipids, and DNA (Fukuoka *et al.* 1990), alteration of vimentin cytoskeleton organization (Kleymenova *et al.* 2005), or membrane alteration in Sertoli cells leading to sloughing of spermatogenic cells (Kleymenova *et al.* 2005). However, the cellular target of DBP and molecular mechanisms of DBP-induced spermatogenic cell apoptosis remain to be unknown.

The study on serum levels of DBP in thelarche patients (a premature breast development before age 8) showed that 28 of 41 (68%) thelarche patients displayed significantly higher (15–276 mg/l) levels of DBP (Colon *et al.* 2000). The high-serum level of DBP in thelarche patients is a matter of concern as an estrogenic xenobiotic. DBP and butyl benzyl phthalate were found to be capable of binding to estrogen receptor α (ERα) and then enhancing the proliferation of MCF-7 human breast cancer cells expressing ERα (Jobling *et al.* 1995, Harris *et al.* 1997, Zacharewski *et al.* 1998, Nishihara *et al.* 2000). In addition, DBP has been shown to exhibit an

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