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Re: Manuscript No. TAAP-D-05-00234

Dear Dr. Waalkes,

Thank you very much for your kind letter dated June 15, 2005, and we highly appreciate the comments from the reviewers as well. Enclosed please find our revised manuscript entitled, "Differential susceptibilities of Holtzman and Sprague-Dawley rats to fetal death and placental dysfunction induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) despite the identical primary structure of the aryl hydrocarbon receptor." by Kawakami T. and coworkers.

We have accepted nearly all the comments by the referees, and performed additional studies including the analyses of TCDD in the tissues and gene expression by microarray. The specifics of our replies are described below.

Reviewer I

Comment 1.

Can you provide evidence that the transfer area of the maternal blood sinusoids is, in fact, reduced in HLZ rats exposed to TCDD in utero? Is the density of sinusoids comparable in the two rat strains? This could have bearing on the potential role of hypoxia in fetal loss although the role of hypoxia is not well supported by the growth data presented previously.

Action taken:

We agree with the referee's comment that the transferring area of MBSs should be provided. We performed morphometrical analysis on HE-stained cross-sections. We have added statistical data in Figure 3, and some statements in Materials & Methods and Results sections.

[After revision]

P.5, Line 19-22,

“Morphometrical measurement of maternal blood sinusoid was carried out using Scion Images software (Scion Corporation, Frederick, MD). Briefly, total area of maternal blood sinusoids was traced in the 0.15-mm² area of LZ in the HE cross-section and then the ratio was calculated. A total of three sections from each group were analyzed.”

P.6, Line 36-38,

“For comparison of maternal blood sinusoids area among HLZ and SD-IGS rats that were administered vehicle or TCDD, one-way analysis of variance (ANOVA) was carried out followed by Fisher’s PLSD as a post-hoc test.”

P.7, Line 35-36,

“Morphometry revealed the significantly larger area of maternal blood sinusoids of vehicle-control HLZ rats than those of vehicle-control SD-IGS and TCDD-treated both rats (Fig 3).”

P17, Line 16-20

“FIG. 3. Morphometry of maternal blood sinusoids. Area of maternal blood sinusoids (MBS) was measured by Scion Image software. The sum of MBS area was divided by total area of observed labyrinth zone (n = 3). HLZ rats were administered 1.6 µg TCDD/kg; SD-IGS rats were administered 10 µg TCDD/kg. Statistically significant difference between means from the vehicle-treated HLZ rat and the other three groups was analyzed by ANOVA followed by Fisher’s PLSD test (* $p < 0.01$).”

Comment 2.

Some discussion of the study timetable should be included. The finding of nonviable fetuses on gestational day 20 implicates prior toxic events. Why, for example, were the assays for induction of mRNAs for CYP1A1 and AhR not performed at stages between day 15 and 20?

Action taken:

We agree with the referee’s comment and compared the gene expression levels of CYP1A1 and AhR levels from the two strains of rats by using real-time RT-PCR. In order to do so, we reexamined placental and fetal liver tissues collected on GD16 and GD20 from SD-IGS and HLZ rats that were injected with TCDD on GD15. We could not detect any difference in the both message levels between HLZ and SD-IGS rats on the two time points as well as the both time points. It is thus concluded that the impact of TCDD exposure shortly after administration did not differ between HLZ and SD-IGS. According to this result, we have added several sentences in the text as described below.

[After revision]

P.9, Line 3-8,

“Using real-time RT-PCR, we determined the induction of AhR and CYP1A1 mRNAs in

placental and fetal liver tissues collected on GD16 and GD20 from SD-IGS and HLZ rats that were injected with TCDD on GD15. We could not detect any difference in the both message levels between HLZ and SD-IGS rats on the both collection time points as well as the two time points. It is thus concluded that the impact of TCDD exposure shortly after administration did not differ between HLZ and SD-IGS rats (data not shown)."

Reviewer II

Comment 1:

The authors have not measured exposure of the rats to TCDD both in the blood and target tissues, the concentration of TCDD in systemic circulation and tissues may be different in the different strains leading to differential toxicity. The authors must have this data to rule out exposure differences.

Action taken:

Since it is extremely laborious and expensive to use high-resolution gas-chromatography attached with high-resolution mass-spectroscopy, we adopted a simplified assay for AhR-lignads, called CALUX. In the present study, due to the administration of TCDD, the total activity of CALUX is considered to reflect TCDD, but not other congeners. We thus determined dioxin amounts in the extracts from maternal serum, placenta, and fetal liver, presented the data in Table 3, and added several sentences in Materials & Methods and Results sections.

[After revision]

P.5, Line 24-26

"CALUX analysis. Chemical-activated luciferase expression (CALUX) assay using H1L1.6.1 mouse hepatoma cell harbouring luciferase reporter gene was done in the Hiyoshi Inc. (Aichi, Japan), based on the previous report (Tsutsumi et al., 2003)."

P.7, Line 38-P.8 Line 4,

"TCDD levels in maternal blood, placenta and fetal liver

To compare the TCDD levels between HLZ and SD-IGS rat tissues, we employed CALUX system using H1L1.6.1 mouse hepatoma cell. From a standard curve using TCDD, we calculated TEQ values in each tissue as indicated in Table 3. Although a higher value was obtained in placenta sample of SD-IGS exposed to 1.6 µg of TCDD/kg, we could not detect much higher values in HLZ rat samples than SD-IGS rat."

P.12, Line 36-37,

"We also thank Mr. Masafumi Nakamura (Hiyoshi Inc., Japan) for his technical assistance in CALUX assay."

P.16, Line 23-25

“36. Tsutsumi, T., Amakura, Y., Nakamura, M., Brown, D. J., Clark, G. C., Sasaki, K., Toyoda, M., Maitani, T., 2003. Validation of the CALUX bioassay for the screening of PCDD/Fs and dioxin-like PCBs in retail fish. Analyst 128, 486-492.”

Comment 2:

The AhR:Arnt complex does cross-talk with many other pathways (ER, hypoxia, etc). Thus the authors should be investigating the possible differential cross-talk between the AhR in these two strains leading to differential susceptibility. Microarrays could assist in illustrating different genes modulated in the different strains. This would give the authors hypothesis some validity. The TCDD:AhR complex functionality tests (1a1, 1b1) may not mediate this toxicity and thus 1 and 2 may assist in differentiating between the toxicity in the two strains.

Action taken:

According to this reviewer's comment, we performed microarray analysis for the two strains of rats and presented the data of scatter plot diagram in Figure 8, and made a new statement for the result.

[After revision]

P.6, Line 27-32,

“Microarray analysis. Filgen Array Rat 27K (Filgen, Inc., Aichi, Japan) spotted 26,962 gene probes was used to analyze expression profiles in placental genes of the HLZ and SD-IGS rats that were exposed vehicle or TCDD. Briefly, the total RNA was converted into T7 promoter connected double strand DNAs and then in vitro transcription was done to amplify the Cy5-labeled cRNAs. The cRNA samples were then hybridized to the Rat 27K Array. The spot intensity was analyzed by GenePix4000B array scanner and GenePix Pro software (Molecular Devices Co., Sunnyvale, CA).”

P.9, Line 18-25,

“Comparison of gene expression profiles in the placenta between HLZ and SD-IGS rats

To compare the gene expression profiles between HLZ and SD-IGS rats, we carried out microarray analysis using total RNAs from vehicle- and 1.6 µgTCDD/kg-treated placentas. We selected 18,077-probes whose ontologies are described in GO database, and then made two Schatter plots comparing signals between vehicle- or TCDD-treated HLZ and SD-IGS (Fig 8). Being consistent with histological results, a much greater difference in mRNA expression profile was detected in vehicle group than TCDD exposed group, indicating that gene expression profile became similar between HLZ and SD-IGS rats after TCDD exposure.”

P.12, Line 21-26,

“In microarray analysis of this study, we detected several genes whose expression levels are higher in HLZ than SD-IGS placenta. The difference of mRNA expression profile in the intact

animals might be involved in different susceptibility between the two rats. Interestingly, opsin 1 (NM_031015) was plotted in both two Schatter plots as a significant outlier gene whose expression level was 13-fold higher in HLZ than SD-IGS both before and after TCDD exposure. This integral membrane receptor might be a candidate that can explain the different susceptibility between the two rats.”

P.18, Line 17-20

“FIG. 8. Microarray analysis of placental transcripts in HLZ and SD-IGS rats. Scatter plot diagram is presented to compare signal levels between HLZ and SD-IGS rats: panel A (vehicle-treated) and panel B (1.6 µg TCDD/kg-treated). Note that a much greater difference was detected in vehicle treated group than compared to TCDD-treated group. Arrowhead indicates, opsin 1 gene. SLR stands for signal log ratio.”

We hope that the revision we have made meets the requirement for publication in Toxicology and Applied Pharmacology.

Thank you for your kind cooperation and understanding.

Sincerely yours,

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Differential susceptibilities of Holtzman and Sprague-Dawley rats to fetal death and placental dysfunction induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) despite the identical primary structure of the aryl hydrocarbon receptor.

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Short title: Different Susceptibility to TCDD in Two Outbred Rat Strains

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Abstract

A single oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) administered to pregnant Holtzman (HLZ) rats on gestational days 15 (GD15) caused placental dysfunction, resulting in fetal death ([Ishimura et](#)

al., 2002a and b). In order to investigate the mechanism underlying the TCDD-induced fetal death, we compared two outbred strains of rats, namely, the HLZ and the Sprague-Dawley International Genetic Standard rats (SD-IGS), a strain with characteristics resembling those of the HLZ rats. Pregnant HLZ and SD-IGS rats were administered TCDD as a single dose by gavage on GD15, as described within the parentheses (HLZ, 0, 1.6 μ g TCDD/kg; SD-IGS, 0, 2, 5, 10 μ g TCDD/kg). Whereas a high incidence (14%) of fetal death was observed on GD20 in the HLZ rats, no fetal deaths occurred in the SD-IGS rats, even at the highest dose of TCDD. A histological marker of cellular abnormality at the placental junctional zone, i.e., delay in the disappearance of the eosinophilic material containing cysts surrounded by glycogen cells glycogen cells and large cysts filled with eosinophilic material (GC-EM), which normally disappear by GD20, was observed in the HLZ rats after exposure to the lowest dose of TCDD (1.6 μ g TCDD/kg), but not in the SD-IGS rats even after exposure to the highest dose of TCDD. Furthermore, maternal blood sinusoids in the labyrinth zone were constricted following exposure to TCDD in the HLZ, but not SD-IGS rats. These observations indicate that HLZ rats are more susceptible to the adverse effects of TCDD on fetal growth and placental function, than SD-IGS rats. Direct sequencing analysis of the aryl hydrocarbon receptor (AhR) gene revealed no difference in the primary structure of the receptor between the HLZ and SD-IGS rats. In addition, no significant differences were observed between the two strains of rats in the levels of induction of placental cytochrome P450 1A1, 1B1, AhR, and AhRR mRNAs following administration of serially increasing doses of TCDD (0.0125, 0.05, 0.2, 0.8 and 1.6 μ g TCDD/kg), indicating that the activity of TCDD-AhR complex in the placenta is similar between the HLZ and SD-IGS both rats. Taken together, the above-described findings indicate that the higher susceptibility of HLZ rats to TCDD-induced placental dysfunction and fetal death may be modulated by other factor(s) in the genetic background of HLZ rats than the AhR.

Key words: Fetal death, Placenta, TCDD, Susceptibility

Introduction

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes a wide variety of adverse effects in laboratory animals (Pohjanvirta and Tuomisto, 1994; Birnbaum et al., 1995; Tohyama C. 2002), and it has been established that the majority of these adverse effects are mediated by an aryl hydrocarbon receptor (AhR)-dependent mechanism. Furthermore, it is well-known that the susceptibility to TCDD differs considerably among animal species: for example, the LD50 of TCDD in hamsters is approximately 8,000-fold higher than that in guinea pigs. (Schwetz et al., 1973; Henck et al., 1981). Even in the same animal species, the C57BL/6J strain of mice and Long-Evans (L-E, *TurkuA/B*) rats show an approximately 10-fold and more than 1,000-fold higher susceptibility to TCDD-induced death than the DBA/2 strain (Chapman and Schiller, 1985) and Han/Wistar (H/W, *Kuopio*) rats (Pohjanvirta et al., 1988; Pohjanvirta et al., 1993), respectively. Differences in the susceptibility to the adverse effects of TCDD among species or even strains of the same species have been ascribed to the distinct primary structures of the AhR in these

animals, since the AhR structure determines its ligand-binding affinity and transactivation function. For instance, the transactivation domain in the C-terminal of the AhR protein is partially deleted in the C57BL/6J mice as compared to that in the DBA/2 mice, and replacement of single amino acid residue (A375V) in the ligand-binding domain causes a difference in the dissociation constant (K_d) (Ema et al., 1994, Poland et al., 1994). Mutation in the transactivation domain in the AhR of H/W rats was demonstrated in one study (Pohjanvirta et al., 1998).

In contrast, it was clearly demonstrated in a series of studies conducted using two experimental animal models (Tuomisto et al., 1999; Smith et al., 1998; Robinson et al., 2002) that susceptibilities to the adverse effects of TCDD are not necessarily always attributable to polymorphism of the *AhR* gene. Tuomisto and coworkers (Tuomisto et al., 1999) cross-bred and generated sublines of H/W and L-E rats, designated as Lines B and C. While both the rat lines had an identical structure of AhR, the LD50 value of TCDD in Line B was significantly higher, approximately 20-fold, than that in Line C, in which the LD50 value of the TCDD was found to be equivalent to that in the L-E rats. These results suggest the possibility of an as-yet unidentified gene (gene B^{hw}) of H/W origin being involved in the differential susceptibility between Lines B and C. By conducting a quantitative trait locus analysis of an F2 intercross between the susceptible C57BL/6 strain and resistant DBA/2 strain, Smith and his associates (Smith et al., 1998; Robinson et al., 2002) reported demonstrated that the differential susceptibility of the animals to hepatic porphyria caused by the administration of iron compounds prior to that of TCDD, was independent of the *AhR* gene loci. These observations suggest the possible existence of so-called modifier genes that determines the toxicity phenotype by modulating AhR gene expression.

Gestational exposure of many species of laboratory animals to TCDD has been established to result in fetal death (Couture et al., 1990, Olson and McGarrigle 1992, Guo et al., 1999). Administration of a relatively low dose of TCDD (1.6 μg TCDD/kg) to pregnant Holtzman (HLZ) rats on gestation days 15 (GD15) was followed by a significantly elevated incidence (13%), the last stage of pregnancy, in the animals (Ishimura et al., 2002a). A novel finding in this study was the considerable delay in the disappearance of the glycogen cells and large cysts filled with eosinophilic material the eosinophilic material containing cysts surrounded by glycogen cells (GC-EM) at the junctional zone of placenta caused by exposure to TCDD. Under normal physiological conditions, glycogen cells appear at around GD13, peak in number around GD16, and disappear by the time of delivery (Davies and Glasser, 1968). These findings suggested that TCDD retarded the normal degradation process of GC-EM and also disrupted the normal glucose kinetics in the placenta.

In our later study, TCDD administration during the gestation period was found to cause constriction of the maternal blood sinusoids in the labyrinth zone of placenta (Ishimura et al., 2003). The junctional zone, located on the uterus side, has been reported to play a role in glycogen storage and hormone secretion (Davies and Glasser, 1968), while the labyrinth zone in which fetal capillaries and maternal blood sinusoids intermingle, plays a role in the transport of oxygen and nutrients from the dam to the fetus through the trophoblastic cell layers. Our earlier observation of the appearance of protein markers of hypoxia suggested the possible involvement of placental hypoxia in the late stage of pregnancy in the toxic effects of TCDD on

the fetus (Ishimura et al., 2002b).

In the present study, we analyzed the differential effects of TCDD on the fetus and placenta of HLZ rats imported from the United States and Sprague-Dawley International Genetic Standard (SD-IGS) rats introduced to Japan from the United States in 1994. While the SD-IGS rats exhibit close resemblance in characteristics to the HLZ rats, it was found that the SD-IGS rats were much less susceptible to the adverse effects of TCDD than the HLZ rats. We also demonstrated that the two rat strains had an identical AhR structure. These findings suggest that some modulating factors beside AhR may reside in the genetic make-up of the two outbred rats, which accounts for the strain difference in the susceptibility to the adverse effects of TCDD.

Materials and methods

Reagents. 2,3,7,8-TCDD (purity > 99.5%) was obtained from Cambridge Isotope Laboratory (Andover, MA). Biotinylated BS-1 lectin, streptavidin-conjugated with horseradish peroxidase, n-nonane, and corn oil were purchased from Sigma (St. Louis, MO). HistoChoice was procured from Amresco (Solon, OH). Trizol reagent, SuperScript II RNase H-Reverse transcriptase, oligo (dT) 12-18 primer and Platinum *Pfx* DNA Polymerase were obtained from Invitrogen (Carlsbad, CA). TaKaRa Ex Taq polymerase was purchased from Takara Bio (Otsu, Japan). QuantiTect SYBR® Green PCR Master Mix, RNeasy Mini kit, and QIAquick Gel Extraction kit were purchased from QIAGEN (Hilden, Germany). The BigDye terminator cycle sequencing kit was purchased from PE-Biosystems (Foster City, CA). The plasmid pGEM-T Easy vector was obtained from Promega Corp. (Madison, WI). Unless otherwise specified, all the other chemicals and reagents were purchased from Wako Chemical Industries (Osaka, Japan).

Experimental animals. Male and female HLZ rats were purchased from Harlan Co. (Indianapolis, IN). Male and female Sprague-Dawley International Genetic Standard (SD-IGS, Crj; CD(SD)IGS) rats (Mitsumori et al., 2001) were purchased from Charles River Co., (Yokohama, Japan). All the rats were 8 weeks old at the beginning of the experiment. Both strains of rats were housed in the vivarium of the National Institute for Environmental Studies (NIES), and handled with humane care according to the guidelines for animal experiments at the NIES. The animals were maintained in a controlled environment at a temperature of $24 \pm 1^\circ\text{C}$, humidity of $45 \pm 5\%$, and 12/12 h light/dark cycle, and given food and distilled water *ad libitum*. Both strains of female rats in proestrus were allowed to mate 1 : 1 with males overnight, and, if a female was found to have a vaginal plug the following morning, that day was defined as GD0. The pregnant rats were housed individually in clear plastic cages with heat-treated wood chips as bedding.

TCDD treatment. The experimental procedure, from TCDD administration to necropsy, was performed in the Hazardous Chemical Regulated Area at NIES. TCDD was dissolved in corn oil containing 4.0% n-nonane. The vehicle used was made of the same concentration of n-nonane and corn oil. In the initial experiment, pregnant rats were given a single oral dose of TCDD (HLZ, 1.6 μg TCDD/kg; SD-IGS, 2, 5,

and 10 µg TCDD/kg dam's body weight; dams = 5-6) or an equivalent volume of vehicle (control) on GD15. In the second experiment, pregnant rats were given a single oral dose of TCDD at varying concentrations (0.0125, 0.05, 0.2, 0.8, and 1.6 µg TCDD/kg dam's body weight, dam = 1). Prior to the terminal euthanasia on GD20, the dams of both strains were anesthetized with diethyl ether and their blood samples were collected from the celiac artery for clinical pathological examinations. The fetuses and placenta were removed from the uterus, and these tissue (placenta, uterus and fetal liver) samples were immediately frozen in liquid nitrogen and maintained at -80°C until analysis. The maternal liver and uterine myometrium adhering to the placenta were also collected.

Histopathology. The placental tissues were fixed in HistoChoice and embedded in paraffin. Both horizontal and longitudinal sections (5-µm-thickness) were prepared on silane-coated slides and stained with hematoxylin and eosin (HE). The horizontal sections were stained with BS-1 lectin to distinguish the fetal capillary endothelium, as described previously (Laitinen, 1987). Briefly, the sections were incubated with 0.3% H₂O₂ for 10 min to remove endogenous peroxidase, followed by treatment with 0.1 M Tris-HCl buffer (pH7.8) containing 0.1% trypsin in 1 mg/ml CaCl₂ for 10 min. Non-specific binding was blocked with 1% BSA in PBS for 10 min, and then with avidin and biotin for 15 min each. The sections were then incubated with 100 µg/ml biotin-labeled BS-1 lectin for 1 h, followed by incubation with streptavidin conjugated with horseradish peroxidase for 1 h. Specific binding was detected using diaminobenzidine solution. Morphometrical measurement of maternal blood sinusoid was carried out using Scion Images software (Scion Corporation, Frederick, MD). Briefly, total area of maternal blood sinusoids was traced in the 0.15-mm² area of LZ in the HE cross-section and then the ratio was calculated. A total of three sections from each group were analyzed.

CALUX analysis. Chemical-activated luciferase expression (CALUX) assay using H1L1.6.1 mouse hepatoma cell harbouring luciferase reporter gene was done in the Hiyoshi Inc. (Aichi, Japan), based on the previous report (Tsutsumi et al., 2003).

Sequencing. Total RNA was isolated from the maternal livers of both HLZ and SD-IGS rats. cDNA synthesis was conducted as described above. Four sets of primers were designed to cover the entire coding region of AhR (Table 2, Fig. 3A). For amplification of the AhR cDNA fragments, 50 µl of a PCR mixture containing 1.25 units of Platinum Pfx DNA polymerase, 10 x PCR Enhancer Solution, 50 mM MgSO₄, 10 x Pfx DNA polymerase, and 1 µl of the reverse-transcribed cDNA was used. PCR was performed by denaturing at 95°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min. The amplified PCR products were purified with the QIAquick Gel Extraction kit and directly sequenced using the Big Dye terminator cycle sequence kit in the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Real-time RT-PCR. Total RNAs were purified from the placenta, fetal liver, maternal liver, and myometrium with Trizol reagent or RNeasy Mini kit, and the RNA samples (4 µg) were reverse-transcribed

for 50 min at 42°C in 20- μ l volume of a reaction mixture containing 200 units of SuperScript™ II reverse transcriptase and 0.5 μ g of oligo(dT) 12-18 primer, in accordance with the standard protocol of the supplier. The expression levels of placental CYP1A1, CYP1B1, AhR, and AhRR mRNA were determined by a real-time RT-PCR method using a LightCycler rapid thermal cycler system (Roche Molecular Systems, Alameda, CA), in accordance with the manufacturer's instructions. The reaction mixture (20 μ l) contained 0.5 μ l of the reverse transcriptase reaction product described above, QuantiTect SYBR® Green PCR Master Mix, and 2 μ M of each of the primers. The primer sets are shown in [Table 1](#). The denaturation step was performed at 95°C for 15 min, and the PCR was carried out in separate glass capillary tubes over 40 cycles (95°C for 15 sec, 60°C for 20 sec, and 72°C for 10 sec). Detection of the fluorescent products was carried out at the end of the 72°C extension period. To confirm the amplification specificity, the PCR products from each of the primer pairs were subjected to a melting curve analysis, followed by 2% agarose gel electrophoresis. The relative amounts of cyclophilin (CP) mRNA as the internal control were also determined to compensate for variations in the RT-PCR efficiency. The expression levels of CYP1A1, CYP1B1, AhR, and AhRR mRNAs were normalized to the average expression level in the control group. The PCR products were subcloned into the pGEM-T Easy vector and sequenced.

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed as described elsewhere ([Ishimura et al., 2002a](#)). The primer sets and PCR cycle conditions were the same as for the real-time RT-PCR ([Table 1](#)). Briefly, total RNA was purified from the liver and uterus of dams with RNeasy Mini kit, and reverse-transcription was performed using the same protocol as described above. For amplification of the CYP1A1 and CP mRNAs, the PCR mixture (25 μ l) contained 0.625 units of TaKaRa Ex Taq™ polymerase, 10 x Ex Taq buffer, 0.2 mM of each dNTP mixture, 2 μ M primer and 0.5 μ l of the reverse transcribed cDNA. The cycling parameters were as follows: 95°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec for CYP1A1; 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec for CP. The PCR products were separated by 2% agarose gel electrophoresis, subcloned into the pGEM-T Easy vector, and sequenced.

Microarray analysis. Filgen Array Rat 27K (Filgen, Inc., Aichi, Japan) spotted 26,962 gene probes was used to analyze expression profiles in placental genes of the HLZ and SD-IGS rats that were exposed vehicle or TCDD. Briefly, the total RNA was converted into T7 promoter connected double strand DNAs and then in vitro transcription was done to amplify the Cy5-labeled cRNAs. The cRNA samples were then hybridized to the Rat 27K Array. The spot intensity was analyzed by GenePix4000B array scanner and GenePix Pro software (Molecular Devices Co., Sunnyvale, CA).

Statistical analysis. StatView for Windows, version 5.0 (SAS Institute, Cary, NC) was used for the statistical analyses. All the results were represented as the means $\pm$ SE. Data for comparison of the number of fetal deaths were analyzed by the χ^2 test. For comparison of maternal blood sinusoids area among HLZ and SD-IGS rats that were administered vehicle or TCDD, one-way analysis of variance (ANOVA) was carried out followed by Fisher's PLSD as a post-hoc test. Differences in the mean values obtained from the

real-time RT-PCR analysis between the HLZ and SD-IGS rats were compared using Student's *t*-test. Differences were considered to be statistically significant when the *p* value was less than 0.05.

Results

Fetal mortality

In the present study, we first examined the fetal mortality rate of both HLZ and SD-IGS rats following exposure to TCDD. In a previous study, the incidence of fetal death on GD20 following administration of 0.8 and 1.6 µg of TCDD/kg on GD15 to HLZ rats was found to be 3.0% and 12.9%, respectively (Ishimura et al., 2002a). In the present study, the incidence of fetal death (11/81; dead/born) in HLZ rats following exposure to 1.6 µg TCDD/kg body weight was 13.9%, consistent with our previous report. In contrast to the conspicuous susceptibility of HLZ rats to fetal death following exposure to TCDD, no fetal deaths occurred in the SD-IGS rats exposed to 0, 2, 5, and 10 µg/kg of TCDD on GD15. Thus, there was a marked difference in susceptibility between HLZ and SD-IGS rats to TCDD-induced fetal death (Table 2).

Histological examination of the placenta

Junctional zone. We previously reported that GC-EM were found on GD20 in HLZ rats exposed to TCDD, despite the fact that the glycogen cells disappears by the time of delivery in the normal rat placenta (Ishimura et al., 2002a). Since fetal death occurred only in the HLZ rats, and not the SD-IGS rats, we investigated whether the appearance of GC-EM was restricted the HLZ rats. As expected, large GC-EM were observed at the junctional zone of the placenta in HLZ rats treated with TCDD (Fig. 1B), but not in the vehicle-treated placenta (Fig. 1A). In the case of SD-IGS rats, no GC-EM were observed at the placental junctional zone even after exposure to the highest dose of TCDD (Fig. 1D, E, F), clearly indicating that exposure to TCDD did not affect the disappearance of the GC-EM in the SD-IGS rats.

Labyrinth zone. Our earlier studies revealed hypoxic changes in the placenta following administration of TCDD in HLZ rats (Ishimura et al., 2002b); yet another study revealed that the structure of the labyrinth vasculature was severely altered by exposure to TCDD (Ishimura et al., 2003). We hypothesized that the reduced blood flow and oxygen supply to the fetus due to the alterations of the vascular structure in the labyrinth may be the primary reason for an increased risk of fetal death following exposure to TCDD in these rats. In vehicle-control HLZ rats, large maternal blood sinusoids filled with red blood cells were seen (Fig 2A-C), whereas in the HLZ rats treated with TCDD, constriction of the maternal blood sinusoids was observed (Fig 2D-F). While the width of the maternal sinusoids in the vehicle-control SD-IGS rats was much smaller than that in the vehicle-control HLZ rats (Fig 2G-I), no alterations in the width were induced following exposure to even the highest dose of TCDD examined in the study (10 µg TCDD/kg) (Fig 2J-L). Morphometry revealed the significantly larger area of maternal blood sinusoids of vehicle-control HLZ rats than those of vehicle-control SD-IGS and TCDD-treated both rats (Fig 3).

TCDD levels in maternal blood, placenta and fetal liver

To compare the TCDD levels between HLZ and SD-IGS rat tissues, we employed CALUX system using H1L1.6.1 mouse hepatoma cell. From a standard curve using TCDD, we calculated TEQ values in each tissue as indicated in [Table 3](#). Although a higher value was obtained in placenta sample of SD-IGS exposed to 1.6 μg of TCDD/kg, we could not detect much higher values in HLZ rat samples than SD-IGS rat.

Comparison of the primary structure of the AhR open reading frame sequences between HLZ and SD-IGS rats

The nucleotide sequences of AhR cDNA from the liver RNAs of HLZ rats and SD-IGS were determined by direct sequencing of the RT-PCR products amplified by a high-fidelity DNA polymerase. The nucleotide sequences of the AhR mRNA open reading frame from the HLZ and SD-IGS rats were completely identical to each other, suggesting that the primary structure of AhR in both rats were the same ([Fig. 4B](#)). It should be pointed out here, however, that the sequence of the AhR mRNA in these rats was different from the one reported for the Sprague-Dawley (SD) rat AhR mRNA sequence in the public database (NM_013149, XM_346769, U04860, [Elferink and Whitlock, 1994](#)); a single nucleotide change was found at position 2381 nt (T to A), which changed the amino residue (H794Q).

Comparison of the strength of induction of CYP1A1 mRNA in the placenta, maternal liver and uterus exposed to TCDD between HLZ and SD-IGS rats

Although HLZ and SD-IGS rats were found to have an identical AhR primary structure, the possibility was considered of a difference in the AhR activity between these two strains of rats. To examine the difference in the transcriptional activity of AhR between the two rat strains, we investigated the cytochrome P450 1A1 (CYP1A1) mRNA induction levels in the HLZ rats (0 and 1.6 μg TCDD/kg) and SD-IGS rats (0, 2, 5, and 10 μg TCDD/kg) exposed to administered TCDD on GD15. The placenta, maternal liver and uterus collected on GD20 were analyzed by real-time RT-PCR. It was observed that the CYP1A1 mRNA induction expression in the placenta reached its maximum levels in both the HLZ and SD-IGS rats ([Fig. 5A](#)). In the SD-IGS rats, maternal liver and uterus CYP1A1 mRNAs were induced in a dose-dependent manner, with no significant differences in the CYP1A1 mRNA induction levels in the maternal liver between the HLZ and SD-IGS rats ([Fig. 5B, C](#)).

Dose-dependent induction of CYP1A1, 1A2, AhR and AhRR mRNAs in the placenta and fetal liver exposed to low-dose TCDD

To further elucidate the transcriptional activity of AhR, we administered a wide range of doses of TCDD to the HLZ and SD-IGS rats (0, 0.0125, 0.05, 0.2, 0.8, and 1.6 μg of TCDD/kg) on GD15, and analyzed the possible dose-dependent induction of CYP1A1, CYP1A2, AhR and AhRR in the placenta ([Fig. 6 A-D](#)) and fetal liver ([Fig. 6 E-H](#)) collected on GD20. Dose-dependent induction of CYP1A1 mRNA was found in the placenta and fetal liver in both the HLZ and SD-IGS rats. Although the levels of CYP1A1, CYP1B1 and AhRR mRNAs in the HLZ rats were slightly higher than those in the SD-IGS rats, the induction levels of all the three genes were similar in the two strains of rats following exposure to the maximum dose (1.6 μg

TCDD/kg), suggesting that the *in vivo* transcriptional activity of AhR was quite similar in the HLZ and SD-IGS rats. There were no differences in the expression levels of AhR mRNA between the vehicle-treated HLZ and SD-IGS rats under any conditions. “Using real-time RT-PCR, we determined the induction of AhR and CYP1A1 mRNAs in placental and fetal liver tissues collected on GD16 and GD20 from SD-IGS and HLZ rats that were injected with TCDD on GD15. We could not detect any difference in the both message levels between HLZ and SD-IGS rats on the both collection time points as well as the two time points. It is thus concluded that the impact of TCDD exposure shortly after administration did not differ between HLZ and SD-IGS rats (data not shown).

Dose-dependent induction of CYP1A1 mRNA in the maternal liver and uterus exposed to TCDD

We further examined effects of TCDD on the CYP1A1 mRNA induction in organs of the maternal body. Dose-dependent (0, 0.0125, 0.05, 0.2, 0.8, and 1.6 μg TCDD/kg) induction of CYP1A1 mRNA in the maternal liver and uterus on GD20 was investigated by semiquantitative RT-PCR and real-time RT-PCR. The CYP1A1 mRNA induction was found to be induced in a dose-dependent manner following TCDD exposure in both the maternal liver and uterus (Fig. 7). The inducibility of CYP1A1 in the maternal liver and uterus was slightly higher in the HLZ rats than in the SD-IGS rats.

Comparison of gene expression profiles in the placenta between HLZ and SD-IGS rats

To compare the gene expression profiles between HLZ and SD-IGS rats, we carried out microarray analysis using total RNAs from vehicle- and 1.6 μg TCDD/kg-treated placentas. We selected 18,077-probes whose ontologies are described in GO database, and then made two scatter plots comparing signals between vehicle- or TCDD-treated HLZ and SD-IGS (Fig. 8). Being consistent with histological results, a much greater difference in mRNA expression profile was detected in vehicle group than TCDD exposed group, indicating that gene expression profile became similar between HLZ and SD-IGS rats after TCDD exposure.

Discussion

The most noteworthy finding in the present study was that HLZ rats were more susceptible to TCDD-induced placental dysfunction and fetal death than SD-IGS rats, despite the identical structure of AhR between these two rat strains. Strain differences in the susceptibility to the adverse effects of TCDD have hitherto largely been explained by *AhR* polymorphism that alters the ligand-binding affinity and/or transcriptional activity of the AhR molecule (Schmidt and Bradfield, 1996; Ema et al., 1994; Pohjanvirta et al., 1993; Chapman and Schiller, 1985). Among mice, the C57BL/6J strain has been reported to show an approximately 10-fold higher sensitivity than the DBA/2 strain to acute lethality and teratogenicity (Chapman and Schiller, 1985). Comparison of the AhR from C57BL/6J mice and DBA/2 mice revealed a deletion in the C-terminal transactivation domain and a single amino acid substitution (A375V) in the ligand-binding domain, which conferred a different dissociation constant (K_d) of AhR against TCDD (C57BL/6J, K_d = 0.27 nM; DBA/2, K_d = 1.66 nM). Thus, the highest susceptibility of C57BL/6J mice to the

adverse effects of TCDD among the various mouse strains tested so far has been ascribed to the distinct affinity of its AhR for TCDD (Ema et al., 1994). When human AhR was knocked into C57BL/6 mice to replace their own AhR, the humanized mice became much less sensitive to the teratogenic effects of TCDD, supporting the idea that the ligand-binding affinity of AhR plays a crucial role in determining the susceptibility to TCDD insult (Moriguchi et al., 2003). In the case of rats, the present study results demonstrated a marked difference in susceptibility to the adverse effects of TCDD between HLZ and SD-IGS rats. It was, therefore, considered reasonable to hypothesize that the *AhR* of HLZ origin may have a significantly higher affinity to TCDD than that of SD-IGS origin. The nucleotide sequence of the AhR mRNA derived from HLZ rats determined at our laboratory showed 99.9% homology with that of the AhR mRNA of SD rats retrieved from the GeneBank database (Accession numbers: NM_013149, XM_346769, U04860, Elferink and Whitlock, 1994) with a mutation (T2381A) which was translated in the amino acid sequence as a single amino acid residue alteration at the C-terminal domain (H794Q). Interestingly, the determined amino acid sequence of HLZ rats was identical to that of SD rats in another public database (Ensembl, ENSRNOP00000006618; UniProt, P41738), and one point-mutation (V497A) was seen in the H/W rat AhR (Pohjanvirta et al., 1998). However, the present study failed to reveal any difference in the sequence of the AhR open-reading frame between the HLZ strain and SD-IGS rats. This observation was lent further support by the absence of any significant difference in the transcriptional activity of AhR between the HLZ and SD-IGS rats in terms of the induction of CYP1A1 mRNA and two other TCDD-AhR inducible genes (Fig. 6). Since HLZ rats were segregated from SD rats in Harlan Co. (Indianapolis, IN) in 1947, it is possible that the genetic background of the HLZ and SD-IGS rats is very similar. We thus concluded that there was no difference in the TCDD-induced transactivation activity of AhR in the placenta and fetal liver between the HLZ and SD-IGS rats. Regarding a possible reason for this distinct susceptibility to the adverse effects of TCDD between the two strains of rats despite the identical AhR structure, it is plausible to hypothesize that there might be as-yet unidentified factors, or “modifier genes”, that are perhaps responsible for the AhR-dependent signaling pathway, but not for transactivation by the AhR-TCDD complex. Existence of such modulators of TCDD-induced fetal toxicity may explain the differential susceptibility of HLZ rats and SD-IGS rats to TCDD-induced placental dysfunction caused by histopathological alterations in the glycogen cells as well as constriction of maternal blood sinusoids (Ishimura et al., 2003).

The presence of modifier genes in mice and rats has also been suggested in earlier studies. In mice exposed to relatively high doses of TCDD, the hepatic uroporphyrinogen decarboxylase (UROD) activity became markedly inhibited, with massive accumulation of uroporphyrin III (URO) in the liver and development of hepatic porphyria; furthermore, the phenomenon was potentiated by pretreatment with iron-dextran (Greig et al., 1984). Using this experimentally induced hepatic porphyria model, Smith and coworkers (Smith et al., 1998) compared SWR and DBA/2 mice that have an identical *AhR* genotype (*AhR^d*), and found that the uroporphyrin level increased more significantly in the SWR mice as compared to the DBA/2 mice, and that there was a difference in the severity of TCDD-induced hepatic porphyria between these two inbred strains of mice. Using quantitative trait loci (QTL) analysis, they also found that an

identical locus on chromosome 11 was responsible for the *AhR* gene-independent difference in susceptibility in the F2 intercross between C57BL/6J and DBA/2 mice and SWR and DBA/2 mice (Robinson et al., 2002). These studies clearly suggest the presence of 'modifier genes' that may modulate TCDD-induced toxicity.

Among rats, the LD50 value of TCDD for L-E rats was found to be more than 1,000-fold higher than that for H/W rats (Pohjanvirta et al., 1988; Pohjanvirta et al., 1993; Pohjanvirta and Tuomisto, 1994). A series of studies in this laboratory suggested that the differences in the susceptibilities of various strains of rats to TCDD toxicity cannot always be ascribed to polymorphism of the *AhR* gene, although the tolerance of H/W rats was attributable, at least in part, to a single point-mutation in the first nucleotide of intron 10 of the *AhR* gene. On the other hand, three sub-lines, namely, Lines A, B and C were established by cross-breeding H/W with L-E rats, which exhibited distinct sensitivities to TCDD-induced lethality due to wasting syndrome (Tuomisto et al., 1999), and both Lines B and C were found to have an identical *AhR* genotype to that of L-E rats. The LD50 value of TCDD in Line B was 20-fold higher than that in Line C and L-E rats, which strongly suggests that the H/W rats probably harbor a modifier gene (gene *B^{hw}*) accounting for these differences in lethality. Lines B and C also exhibited differential sensitivity to TCDD-induced disorder of the reproductive system in the male offspring, when TCDD was administered *in utero* (Simanainen et al., 2004).

The results of the present study using HLZ and SD-IGS rats suggest that these strains can be used to search for modifier genes that may be associated with not only susceptibility to fetal death and placental dysfunction, but also with other endpoints, such as epididymal malformations in offspring perinatally exposed to TCDD (Wilker et al., 1996; Ohsako et al., 2002). These latter malformations have been found in SD-IGS rats, but not in other strains, including HLZ and L-E rats (Mably et al., 1992; Gray et al., 1995; Ohsako et al., 2001). The irreversible adverse effects of TCDD occurring as a result of *in utero* exposure of rat offspring to the TCDD have been shown to differ among different rat strains, depending presumably upon genetic traits other than the *AhR*. Identification of the genes that may be involved in the susceptibility to xenobiotics is important not only for health risk assessment of environmental contaminants, but also for manufacture of drugs. Thus, utilization of these two rat strains is expected to be useful for future toxicological and pharmaceutical studies.

Exposure to TCDD has been reported to increase the fetal mortality in several laboratory animals (Couture et al., 1990; Olson and McGarrigle 1992; Guo et al., 1999), but the precise mechanism underlying this increased mortality is not yet clearly understood. The findings of our current study are consistent with those of previous studies, in that maternal administration of a relatively low dose of TCDD (1.6 µg TCDD/kg) on GD15 in HLZ rats resulted in a high incidence (12.9%) of fetal death on GD20, that is, the last stage of pregnancy, and GC-EM, which normally disappear by GD20, were still observed at the junctional zone of the placenta in these HLZ rats. (Ishimura et al., 2002a). Increased fetal mortality due to placental dysfunction has been suggested in the case of diabetes mellitus (Prager et al., 1974; Gewolb et al., 1986) and alcohol administration in dams (Padmanabhan, 1985). The placental tissues of these dams exhibited similar findings, namely, alterations of the glucose kinetics and delay in the disappearance of

GC-EM, to those observed in the TCDD-exposed rats in the present study. It is thus plausible to speculate that alterations of glucose kinetics may play a common role in these disease conditions as well as in TCDD-induced placental dysfunction. The constriction of maternal blood sinusoids in the labyrinth zone found in cross-sections of the placental tissue derived from TCDD-exposed dams is thought to result in a decrease of the maternal blood flow and reduced supply of nutrients, such as glucose or glycogen cells, from the mother to the fetus. It is not yet clear whether the constriction of the maternal blood sinusoids is caused by alterations in the maternal side or the fetal side, or both. Experiments conducted with exchange of embryos between the HLZ and SD-IGS rat strains might give further insight into this question.

An earlier report (Jana et al., 1998) on the gene expression of CYP1A1 in the liver of male HLZ rats and male SD rats following exposure to TCDD at the dose of 40 ng/kg showed that the HLZ rats exhibited a more than 30-fold higher expression of CYP1A1 mRNA than the SD rats. In contrast, no significant difference in CYP1A1 expression in the liver was observed between pregnant HLZ and SD-IGS rats on GD20. A significantly higher induction of CYP1A1 mRNA was observed in the uterus of HLZ rats than in that of SD-IGS rats following exposure to TCDD, which may explain the difference in the fetal mortality following exposure to TCDD between these two strains of rats (Fig. 7). However, this working hypothesis was excluded by the experiment in which SD-IGS rats administered higher doses of TCDD (SD-IGS, 2, 5, and 10 µg TCDD/kg vs. HLZ, 1.6 µg TCDD/kg) showed identical levels of CYP1A1 mRNA to those in HLZ rats administered TCDD, with no occurrence of fetal death in the SD-IGS rats. The differences in the CYP1A1 expression patterns in the adult liver and uterus might be attributable to a difference of sex, strain (SD-IGS vs. SD), and/or tissue specificity.

In microarray analysis of this study, we detected several genes whose expression levels are higher in HLZ than SD-IGS placenta. The difference of mRNA expression profile in the intact animals might be involved in different susceptibility between the two rats. Interestingly, opsin 1 (NM_031015) was plotted in both two scatter plots as a significant outlier gene whose expression level was 13-fold higher in HLZ than SD-IGS both before and after TCDD exposure. This integral membrane receptor might be a candidate that can explain the different susceptibility between the two rats.

In conclusion, the present study demonstrated that HLZ and SD-IGS rats show differential susceptibility to TCDD-induced placental dysfunction and fetal death, despite showing an identical sequence of AhR, and also very similar transcriptional activities of the AhR in terms of CYP1A1 induction. A search for the possible existence of modifier genes that may explain the strain differences in the susceptibility to TCDD toxicity between the HLZ and SD-IGS rats is warranted in further studies, to obtain useful information for developing tools for health risk assessment of xenobiotic chemicals in the future.

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Figure legends

FIG. 1. Administration of TCDD on GD15 resulted in histopathological alterations in the junctional zone of the placenta on GD20 in the HLZ, but not SD-IGS rats. Hematoxylin and eosin staining. Vehicle-treated HLZ (A) and SD-IGS (C) rats; TCDD-exposed HLZ rats (B, 1.6 μ g TCDD/kg) and SD-IGS rats (D, 2 μ g TCDD/kg; E, 5 μ g TCDD/kg; F, 10 μ g TCDD/kg). JZ, junctional zone; LZ, labyrinth zone; asterisk, GC-EM. Bar = 250 μ m.

FIG. 2. Administration of TCDD on GD15 resulted in histopathological alterations in the labyrinth zone of the placenta in the HLZ, but not SD-IGS rats. Cross-sections (5- μ m thickness) of the placenta were stained with hematoxylin and eosin (A, B, D, E, G, H, J, K), and the fetal capillary cells were visualized by BS-1 lectin staining (C, F, I, L). A-C, vehicle-treated HLZ; D-F, HLZ rats administered 1.6 μ g TCDD/kg; G-I, vehicle-treated SD-IGS; J-L, SD-IGS rats administered 10 μ g TCDD/kg. Asterisks, maternal blood sinusoid; arrows, nuclei of trophoblast cells. Bar = 100 μ m.

FIG. 3. Morphometry of maternal blood sinusoids. Area of maternal blood sinusoids (MBS) was measured by Scion Image software. The sum of MBS area was divided by total area of observed labyrinth zone ($n = 3$). HLZ rats were administered 1.6 μ g TCDD/kg; SD-IGS rats were administered 10 μ g TCDD/kg. Statistically significant difference between means from the vehicle-treated HLZ rat and the other three groups was analyzed by ANOVA followed by Fisher's PLSD test (* $p < 0.01$).

FIG. 4. Primary AhR structure in the HLZ and SD-IGS rats. (A), AhR molecular structure, and position of primer sets used for the RT-PCR. The four lines on the bottom represent RT-PCR products and the arrows indicate the primers listed in Table 1. (B), Partial nucleotide sequences obtained in this study and compared with a sequence retrieved from GenBank. No difference was observed in the primary structure of AhR between the HLZ and SD-IGS rats. However, the AhR sequence of SD-IGS rats was found to be different from the one deposited in a public database (NM_013149, XM_346769, U04860, [Elferink and Whitlock, 1994](#)); mutation at position 2381 nt (T to G) with change of a single amino residue (H794Q).

FIG. 5. CYP1A1 mRNA levels in the placenta (A), maternal liver (B) and uterus (C) of HLZ and SD-IGS rats exposed to TCDD. Pregnant rats were administered TCDD orally by gavage (HLZ; 0, 1.6 μ g TCDD/kg, SD; 0, 2, 5 or 10 μ g TCDD/kg) on GD15, and tissue specimens were collected on GD20 ($n = 3$). The CYP1A1 mRNA level in each tissue specimen was determined by real-time RT-PCR and normalized as a ratio to the corresponding cyclophilin (CP) mRNA level.

FIG. 6. mRNA levels of CYP1A1 (A and E), CYP1B1 (B and F), AhR (C and G), and AhRR (D and H) in the placenta (A-D) and fetal liver (E-H) of rats exposed to TCDD. The HLZ and SD-IGS rats were exposed to TCDD at doses of 0, 0.0125, 0.05, 0.2, 0.8 or 1.6 μ g TCDD/kg on GD15, and placenta and fetal

liver specimens were collected on GD20 ($n = 3$). The mRNA levels of the four genes were determined by real-time RT-PCR and normalized as ratios to the cyclophilin mRNA level. The different *Y*-axis scales in the CYP1A1 and CYP1B1 inset represent doses in the range of 0 to 0.2 μg TCDD/kg. Differences in the mean values between the HLZ and SD-IGS rats were statistically evaluated by Student's *t*-test ($*p < 0.05$, $**p < 0.01$).

FIG. 7. CYP1A1 mRNA levels in the maternal liver and uterus of HLZ and SD-IGS rats exposed to various doses of TCDD (0, 0.0125, 0.05, 0.2, 0.8 or 1.6 μg TCDD/kg) on GD15; the maternal liver and uterus specimens were collected on GD20. (A) Agarose gel patterns of PCR products of the maternal liver and uterus from each TCDD dose group are shown. (B) Real-time analysis for CYP1A1 mRNA levels in the uterus ($n = 3$). Relative mRNA levels are represented as ratios to the cyclophilin mRNA level. Note that the CYP1A1 mRNA levels in the HLZ rat maternal liver and uterus tended to be higher than those in the SD-IGS rats. The different *Y*-axis scales in the CYP1A1 inset represent doses in the range of 0 to 0.2 μg TCDD/kg. Differences in the mean values between the HLZ and SD-IGS rats in the real-time RT-PCR analysis were compared using Student's *t*-test ($*p < 0.05$).

FIG. 8. Microarray analysis of placental transcripts in HLZ and SD-IGS rats. Scatter plot diagram is presented to compare signal levels between HLZ and SD-IGS rats: panel A (vehicle-treated) and panel B (1.6 μg TCDD/kg-treated). Note that a much greater difference was detected in vehicle treated group than compared to TCDD-treated group. Arrow indicates, opsin 1 gene. SLR stands for signal log ratio.

TABLE 1. Primers used for Real-time RT-PCR and Direct Sequencing.

Genes	Primer Sequences		Product size (bp)	Anneal Temp (°C)	GenBank accession no.
	Forward	Reverse			
Cyclophilin ^a	5'-GGAATGGCAAGACCAGCAAG-3'	5'-AGGGTATTGCGAGCAGATGG-3'	141	56	BC059141
CYP1A1	5'-TTTGTCCCATTCACCATCCC-3'	5'-GTCTCCCCAATGCACTTTCG-3'	341	56	NM_012540
CYP1B1	5'-CATCCTCGCTCATCAGTGCAAT-3'	5'-CATTTCAGTTACCTCCGGCCTC-3'	208	56	NM_012940
AhR	5'-GAAGCACATGCAAGTCAATGGC-3'	5'-GACTGGTTGCAGGGAGCAAAGT-3'	192	56	NM_013149
AhRR	5'-ACACCGTGGGCATGGTATACTG-3'	5'-GGATGCTGACTTTCACGTGGC-3'	159	56	AY367561
AhR ^b	5'-ATGAGCAGCGGCGCCAACATCACCTA-3'	5'-TCATGCGGATGTGGGATTCTGCGCAG-3'	1015	57	NM_013149
	5'-CCGTCCATCCTGGAAATTCGAAC-3'	5'-CGTCCTGTTGGATCAAGGCACTC-3'	571	57	
	5'-TTCCCTTATGAGTGCCTTGATCCAAC-3'	5'-CAGGGAAGTCCAAGTGTGTACCCCTTG-3'	827	57	
	5'-CTCAGCAGGAACGAAAGCAC-3'	5'-CCCTCACAGTTCTGGTGTCTGTTCC-3'	605	57	

^a Primer sets for Real-time RT-PCR analysis.
^b Primer sets for direct sequencing of open reading frame of AhR cDNA.

TABLE 2. Effect of GD15 TCDD-Exposure on Reproductive Outcomes on GD20

	Maternal TCDD dose ($\mu\text{g/kg}$)					
	Holtzman		Sprague-Dawley			
	0	1.6	0	2	5	10
Number of dams	5	6	5	5	5	5
Number of total fetuses	73	81	71	71	67	69
Number of fetuses per litter ^a	14.6 $\pm$ 0.76	13.5 $\pm$ 0.93	14.2 $\pm$ 0.92	14.2 $\pm$ 0.97	13.4 $\pm$ 0.81	13.8 $\pm$ 0.86
Total number of dead fetuses	0	11*	0	0	0	0
Number of dead fetuses per litter ^a	0	1.83 $\pm$ 1.0	0	0	0	0
Percent of dead fetuses per litter ^a	0	13.9 $\pm$ 7.4	0	0	0	0

^a Values represent means $\pm$ SE for the letters of five or six dams.

* The statistical significance of difference from vehicle-treated controls was evaluated by χ^2 test ($p < 0.01$).

TABLE 3. TCDD concentrations measured by CALUX assay

	Holtzman			Sprague-Dawley		
	Vehicle	0.2 µgTCDD/kg	1.6 µgTCDD/kg	Vehicle	0.2 µgTCDD/kg	1.6 µgTCDD/kg
Maternal serum	ND (< 6.1*)	ND (5.3)	99.1	ND (< 6.1)	16.5	74.1
Placenta	ND (< 8.8)	ND (3.7)	80.7	ND (< 8.1)	11.2	200.3
Fetal liver	ND (< 3.3)	6.8	75.7	ND (< 3.3)	15.2	62.4

* Data was presented as pgTEQ/g wet tissue.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.