# PROTEOMICS ANALYSIS OF PLACENTAS AFTER EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) IN RATS

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## Introduction

Exposure to 2,3,7,8-TCDD during pregnancy is often observed to cause fetal death in many animal species. Although the fetal death is a very important feature of TCDD toxicities, the exact mechanism is largely unknown. In our earlier work we analyzed the placenta from TCDD-exposed rats and demonstrated an alteration of placental glucose kinetics after exposure to TCDD[1]. In brief, exposure of pregnant Holtzman rats to 1600 ng TCDD/kg resulted in an increase in fetal death on gestational day (GD) 20, and the histology of TCDD-exposed placenta on GD20 showed a larger area occupied by both glycogen cells and cysts, the latter of which was filled with eosinophilic material (GC-EM). In addition, increases in glycogen content and glucose transporter 3 (GLUT3) mRNA level were observed in TCDD-exposed placenta. On the other hand, diabetic rats, which showed the intrauterine growth retardation, have been known to exhibit changes in placental histology or gene expression similar to TCDD-exposed placenta. The placentas of diabetic rats showed a larger GC-EM area[2, 3], increased glycogen content[3, 4], and increased GLUT3 mRNA level[5]. Under physiological condition, large quantities of blood that are retained in the placenta are supplied to fetuses to sustain their growth, and thus the placental hypoxia caused by a decrease in placental blood flow is thought to be one of the most plausible causes of intrauterine fetal death. In the diabetic placenta, the quantities of uterine artery blood flow to the placenta were decreased in volume in the late pregnancy compared to that of normal placenta[6-8]. Therefore, we hypothesized that TCDD-exposed placenta is under hypoxic condition, which may be responsible for placental changes found in TCDD-exposed rats.

In the present study, in order to clarify mechanisms underlying the changes in TCDD-exposed placenta, we analyzed the placental proteins, by two-dimensional electrophoresis (2D/E), of the placental tissues not only from the TCDD-exposed rats but also from the uterine-artery ligation rat model, which mimics the hypoxia in both placenta and fetuses.

## Material and Methods

*Animals and sample collection*

Animal experiments were performed according to the guidelines on animal welfare at NIES. The protocol for TCDD administration was essentially the same as described previously[1]. Briefly, Holtzman rats were given a single oral dose of 1600 ng TCDD/kg body weight or an equivalent volume of vehicle (control) on GD15. The placentas were collected on GD16 and GD20 (n=4 pregnant rats for both control and TCDD groups). The uterine artery ligation model was prepared by the surgery through a midline abdominal incision on GD15. The uterine artery was ligated by placement of a single silk ligature around the cervical end of the uterine artery that supplies right uterine horn. The left uterine

horn was left untouched to obtain control placentas. On GD20, the placentas of live fetuses were collected. The placentas were immediately frozen in liquid nitrogen, and kept at –80 °C until analyzed.

*Two-D/E analysis*

The placental tissue was homogenized with 10 mM Tris-HCl (pH 7.4) buffer, containing 150 mM NaCl and protease inhibitors (complete EDTA-free: Roche Diagnostic, Mannheim, Germany). After centrifugation at 15,000 x *g* for 10 min at 4 °C, the supernatant was collected. The proteins were analyzed by 2D/E as described previously [9], and the protein spots were visualized by silver staining. The intensity of the protein spots was evaluated quantitatively and compared among the 2D/E gels using Scion image analysis software (Beta 4.0.2).

*Protein collection and sequence analysis*

The proteins were collected by using the preparative 2D/E method. To identify the primary structure of these purified proteins, amino acid sequence analysis was performed for each fragmented peptide that was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) after enzymatic digestion [9].

**Results and Discussion**

The 15,000 x *g* supernatant fractions of placental homogenates were prepared and applied to the 2D/E. Gel images were compared between control and TCDD groups, and amounts of eight protein spots were found to be changed after exposure to TCDD (p1, p2, p3, s1, s2, s3, s4, and s5). On GD16, exposure to TCDD increased the amounts of proteins s1, s2, and s4 significantly, tended to increase the amounts of p1, p2 and s5 without a significant difference, and did not change the amounts of p3 and s3. On GD20 the amounts of all the eight proteins p1-p3 and s1-s5 were increased significantly approximately from 1.5 to 3-fold by TCDD-exposure.

Since nearly no information was available about the marker genes of placenta under the hypoxic condition, we tried to characterize the proteins which are relevant to the hypoxia. Thus, we ligated a uterine artery of the rats to exert hypoxic condition by reduction of placental blood flow and analyzed the protein expression by 2D/E. No changes in the amounts of p1-p2 or s1-s5 were found between control and uterine-artery ligated placentas on GD20 whereas the amount of p3 was increased approximately 2-fold by uterine-artery ligation. These results indicated that the increased amount of p3 protein was under the regulation of hypoxia in placenta.

By the use of internal amino acid sequence analysis we found that the p1, p2, and p3 proteins are ß-tropomyosin (ß-TM), heat shock protein 27 (Hsp27), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. These proteins have been thought to be expressed in the placental trophoblast cells. On the other hand, the s1, s2, and s3 proteins were found to be haptoglobin, s4 is c-reactive protein, and s5 is apolipoprotein M. It was reported that the haptoglobin and c-reactive protein, both of which were synthesized mainly in the liver, were induced in amounts and secreted into blood circulation by subacute reaction to infection [10]. It is thus reasonable to speculate that TCDD administration to the pregnant rats increased the levels of these serum proteins, and those detected by the 2D/E analysis in the present study from the maternal serum that were abundantly present in the placental tissue specimens.

In the present study, we found that the amount of GAPDH protein in placenta was increased by the uterine-artery ligation. It was reported that the expression of GAPDH was increased under the hypoxic condition [11-13], and that the hypoxia response element (HRE) is located in the promoter region of GAPDH gene [14]. Thus, the present study demonstrated for the first time that the GAPDH may reflect the hypoxic condition of placenta. The amount of GAPDH protein was shown to increase in the

placenta after exposure to TCDD. Therefore, this is the first study showing that the TCDD-exposed placenta on GD20 was in the hypoxic condition. We previously observed the increase of GLUT3 mRNA in TCDD-exposed placenta on GD20 [1]. Since the expression of GLUT3 gene is known to be regulated by the hypoxia [12], increased GLUT3 mRNA level supported the notion that the TCDD-exposed placenta was under hypoxic condition. We also observed that the amounts of Hsp27 or b-TM proteins were increased in TCDD-exposed placenta, but not by uterine-artery ligation, suggesting that the expression of these proteins is regulated by other factors than the hypoxia.

In summary, we clarified that amounts of Hsp27, b-TM, and GAPDH proteins were increased in the placentas after exposure to TCDD by 2D/E analysis. In addition, the analysis of uterine-artery ligation model strongly suggests that the amount of GAPDH protein was useful as a marker protein to monitor the placental hypoxia. Although the underlying mechanism of the hypoxia in TCDD-exposed placenta is largely unknown, it may be similar to the hypoxic condition in rat diabetic model. Finally, the increased risk for fetal death which occurred after TCDD-exposure might be partly due to hypoxia in the placenta.

**References**

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