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Teratological effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): induction of cleft palate in the ddY and C57BL/6 mouse

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a highly toxic halogenated aromatic hydrocarbon, is a teratogen to induce cleft palate when exposed during the pregnancy. There are inter-strain differences in the sensitivity to cleft palate induced by TCDD and other chemicals including polychlorinated terphenyls (PCTs). The C57BL/6 mouse and the ddY mouse had been shown to be different in the induction of cleft palate following the treatment of PCTs, which attempts us to evaluate the TCDD-induced cleft palate in two mouse strains to understand the mechanism through which TCDD and PCTs induce cleft palate. This study evaluated the induction of cleft palate in the fetuses of ddY and C57BL/6 mice after subcutaneous treatment of TCDD on gestation day (GD) 10.5-14.5 or oral treatment on GD 8.5-13.5. Our results clearly showed that ddY mice, a susceptible strain to PCTs-induced cleft palate, are resistant to the induction of cleft palate by TCDD comparably to the high susceptibility of C57BL/6 mice, suggesting a different teratological mechanism between TCDD and PCTs. In addition, at the low doses, our study supported the concept of "window effect" of TCDD on around GD 12 for the induction of cleft palate in C57BL/6 and ddY mice.

Key words: cleft palate, ddY mouse, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a member of halogenated aromatic hydrocarbons, is a widely spread environmental contaminant [26]. TCDD has a variety of adverse biological effects including carcinogenesis, immune and hemopoietic dysfunction, neuronal cell damage, teratogenesis and reproductive toxicity [13, 18, 19, 24]. The induction of cleft palate is known to be a sensitive teratological effect of TCDD when animals are exposed to TCDD during the pregnancy [10, 21].

Many mouse strains have been used for toxicological and pharmacological studies. Sometimes, the use of mouse strains with different characteristics provides an important clue to approach the toxic and pharmacological mechanism of chemicals. In association with TCDD, DBA/2, a mouse strain with a mutation on the AhR locus of DNA, has been used to investigate the toxic mechanism of TCDD [8, 19, 22, 23]. Compared with the TCDD-sensitive C57BL/6 mice, the resistance of DBA/2 mice to TCDD-induced toxicity had suggested that AhR is involved in the toxic mechanism of TCDD. Later, it had been proved by AhR knock-out mice that the toxic effects of polyhalogenated aromatic compounds including TCDD are mediated by the AhR [20].

The polychlorinated terphenyls (PCTs) having a similar chemical structure to polychlorinated biphenyl (PCB) that is a member of polyhalogenated aromatic compounds has also been shown to induce cleft palate [17]. However, the mechanism by which PCTs induce cleft palate is still speculative. Kaneko and his college used C57BL/6 and ddY mice to investigate the teratological effect of PCTs and its mechanism [17]. It is interesting in their report that C57BL/7 mouse strain sensitive to TCDD-induced cleft palate was resistant to PCTs-induced cleft palate. On the other hand, ddY mice showed high incidence of cleft palate following PCTs treatment. On the basis of the previous study, we hypothesized that those two mouse strains would show different susceptibility to TCDD-induced cleft palate, of which the confirmation would be helpful to extend our understanding in the teratological mechanisms of TCDD and PCTs.

For that purpose, in the present study, we evaluated the induction of cleft palate in ddY and C57BL/6 mice after subcutaneous or oral treatment of TCDD during the pregnancy and compared. Our results clearly showed that, unlike the cleft palate induced by PCTs treatment, the ddY mouse was resistant to TCDD-induced cleft palate comparably to the high susceptibility of C57BL/6 mice,

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which strongly suggested that TCDD and PCTs give rise to their teratological effect by different mechanisms.

Materials and Methods

Chemicals

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Radian International, Cambridge Isotope Laboratories, Inc., Andover, MA, USA, and its purity was 98 %. TCDD was initially dissolved in a small volume of acetone and subsequently adjusted to a working concentration in olive oil.

Animals

Female and male C57BL/6 and ddY mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) at 6-8 weeks of age and held for 2 weeks prior to mating. Two females were housed overnight with one male and checked the presence of a vaginal plug in the next morning, denoted as gestation day 0.5 (GD 0.5). The plug-positive females were maintained in a vinyl isolator established in the hazard room to prevent an environmental exposure. The room was kept under the conditions of $22 \pm 1^{\circ}C$ in temperature, $50 \pm 10\%$ in humidity and 12/12 light/dark cycle. During the study, the mice were given food (CRF-1, Oriental Yeast Co. LTD) and water *ad libitum*.

Treatment and experimental design

For this study, two different administration routes were chosen, subcutaneous (SC) and oral (PO). The doses were selected on the basis of the results of previous studies [7] and our preliminary studies. C57BL/6 and ddY mice were respectively given a single dose of 0, 20, 40 and 80 μg TCDD/kg bw in 10 ml olive oil/kg bw by subcutaneous injection on GD10.5, 11.5, 12.5, 13.5 and 14.5. For the oral study, a single dose of 0, 10, 20 and 40 μg TCDD/kg bw for C57BL/6 mice and 0, 20, 40 and 80 μg TCDD/kg bw for ddY mice was given by gavage on GD8.5, 9.5, 10.5, 11.5, 12.5 and 13.5, respectively. Five pregnant mice per group were used, but the number was sometimes decreased because of non-pregnancy. On GD18, the dams were killed by decapitation. The number and position of all fetuses, live and dead, and of resorptions were noted. Live fetuses were grossly examined to evaluate the incidence of cleft palate, and then fixed in 10% neutral buffered formalin. For histological examination, the sections of craniofacial tissues were processed, embedded in paraffin and stained with hematoxylin and eosin (H&E).

Data analysis

The litter was considered the basic experimental unit. The Kruskal-Wallis test was used to assess the analysis of variance. The significance of the dose-response trend was determined using Jonckheere's test against ordered alternatives, and when this test indicated a significant trend, pairwise comparisons were made using the Mann-Whitney *U* test [14]. The magnitude of the right-left severity score difference for cleft palate was assessed using the Wilcoxon matched-pairs signed-ranks test [6].

Results

Fetal mortality and indcidence of cleft palate

C57BL/6 mice (Table 1, 3) : Four doses of TCDD (0, 20, 40 and 80 μg/kg bw) were singly injected subcutaneously on GD10.5, 11.5, 12.5, 13.5 and 14.5. No effects of TCDD at these concentrations, when injected subcutaneously, were seen on fetal mortality irrespective of the gestation days injected. The oral treatment of 20 μg TCDD/kg bw did not give any effect on the fetal mortality. However, when 40 μg/kg bw of TCDD was orally administered on GD8.5, the percentage of fetuses dying at the late stage was significantly high (31%). In C57BL/6 mice, TCDD clearly induced cleft palate, which was depending on the concentration and the gestation day when TCDD was injected. When 20 μg/kg bw of TCDD was subcutaneously injected, the incidence of cleft palate was observed in the fetuses exposed to TCDD only on GD 12.5 and 14.5 although its rate was very low. The incidence of cleft palate, when 40 μg/kg bw of TCDD was subcutaneously injected, was significantly high in the fetuses exposed to TCDD only on GD 12.5, indicating the "window effect" of TCDD on the induction of cleft palate. However, the subcutaneous treatment of 80 μg/kg bw of TCDD highly induced cleft palate at all TCDD-injected GD points except for GD 14.5.

When TCDD was administered orally, 20 μg/kg bw of TCDD failed to give an effect on fetal mortality in C57BL/6 mice. However, an increase in the number of fetuses dying at the late stage was noted when 40 μg/kg bw of TCDD was administered on GD 11.5. The teratological effect of TCDD was clear in the incidence of cleft palate when 10 μg/kg bw of TCDD was orally given on GD 11.5 and 12.5; 37.5 and 27.8%, respectively. The increase of dose to 20 μg/kg bw not only highly increased the incidence of cleft palate on GD11.5 and 12.5, but also induced cleft palate even on GD8.5, 9.5, 10.5 and 13.5. The oral treatment of 20 μg TCDD/kg bw on GD11.5 and 12.5 induced cleft palate in most of the fetuses (>94 %), and the incidence rates of cleft palate were respectively 20, 26.7, 69.1 and 35.3% when treated on GD8.5, 9.5, 10.5, and 13.5. The oral treatment of 40 μg TCDD/kg bw on GD8.5 - GD12.5 was enough to induce cleft palate in all of the fetuses, and on GD13.5 half of the fetuses were affected.

ddY mice (Table 2, 4) : When TCDD was treated subcutaneously, there were no statistically significant effects of TCDD at the concentrations of 20, 40 and 80 μg

Table 1. Fetal mortality and induction of cleft palate in C57BL/6 mice following subcutaneous treatment of TCDD during pregnancy

Group (g/kg)		GD GD 10.5 (%)[a]	GD 11.5 (%)	GD 12.5(%)	GD 13.5 (%)	GD 14.5 (%)
	No. of mother	4	5	3	4	5
	No. of fetus	29	37	25	25	36
0	No. of early died fetus	0	1(2.7)	2(8.0)	0	2(5.56)
	No. of late died fetus	1(3.45)	1(2.7)	0	1(4.0)	0
	No. of fetus with CP	0	0	0	0	0
	No. of mother	2	5	5	2	2
	No. of fetus	14	38	30	16	17
20	No. of early died fetus	3(21.43)	2(5.26)	3(10.0)	2(12.5)	4(23.53)
	No. of late died fetus	0	0	0	0	0
	No. of fetus with CP	0	0	1(3.33)	0	1(5.88)
	No. of mother	5	5	4	4	2
	No. of fetus	41	44	32	30	17
40	No. of early died fetus	1(2.44)	1(2.27)	2(6.25)	4(13.33)	2(17.76)
	No. of late died fetus	0	0	0	0	0
	No. of fetus with CP	1(2.44)	0	8(25.0)*	0	0
	No. of mother	4	4	4	3	3
	No. of fetus	44	28	33	18	24
80	No. of early died fetus	1(2.27)	3(10.71)	4(12.12)	0	2(8.33)
	No. of late died fetus	0	0	0	1(5.56)	0
	No. of fetus with CP	15(34.09)*	8(28.6)*	9(27.3)*	5(27.8)*	0

[a]%of affected fetuses/total live fetuses

*$p<0.05$ vs control

Table 2. Fetal mortality and induction of cleft palate in ddY mice following subcutaneous treatment of TCDD during pregnancy

Group (g/kg)		GD GD 10.5 (%)[a]	GD 11.5 (%)	GD 12.5(%)	GD 13.5 (%)	GD 14.5 (%)
	No. of mother	5	4	5	5	4
	No. of fetus	54	51	56	60	38
0	No. of early died fetus	2(2.37)	0	1(1.78)	2(3.33)	1(2.63)
	No. of late died fetus	1(1.85)	1(1.96)	0	1(1.67)	2(5.26)
	No. of fetus with CP	0	0	0	0	0
	No. of mother	5	5	3	4	4
	No. of fetus	57	61	25	54	36
20	No. of early died fetus	3(5.26)	3(4.92)	3(12.5)	1(1.85)	8(22.2)
	No. of late died fetus	1(1.75)	1(1.64)	0	1(1.85)	2(5.56)
	No. of fetus with CP	0	0	0	0	0
	No. of mother	5	4	5	4	5
	No. of fetus	52	48	65	58	62
40	No. of early died fetus	2(3.85)	3(6.25)	4(6.15)	1(1.72)	1(1.61)
	No. of late died fetus	1(1.92)	0	1	1(1.72)	0
	No. of fetus with CP	0	0	0	1(1.72)	1(1.61)
	No. of mother	5	4	5	5	4
	No. of fetus	57	51	42	57	48
80	No. of early died fetus	5(8.77)	2(3.92)	4(9.52)	7(12.28)	0
	No. of late died fetus	0	2(3.92)	0	3(5.26)	1(2.08)
	No. of fetus with CP	0	0	0	0	0

[a]%of affected fetuses/total live fetuses

/kg bw on fetal mortality irrespective of the gestation days injected. However, when 80 μg/kg bw of TCDD was orally administered on GD 10.5, 13 fetuses from two dams died at the late stage of gestation; 9/12 and 4/15,

respectively. Twelve fetuses from three dams treated with 80 μg/kg bw of TCDD on GD 13.5 died at the early stage of gestation (7/11, 2/11, and 3/14).

Compared with C57BL/6 mice, ddY mice were very resistant to the teratological effect of TCDD in the induction of cleft palate. When TCDD was injected subcutaneously, cleft palate didn`t occur even at the concentration of 80 μg/kg bw. Only one fetus that 40 μg/kg bw of TCDD was subcutaneously injected on GD 13.5 had cleft palate. The ddY mouse also showed a prominent resistance to the induction of cleft palate following the oral treatment of TCDD. In our study, while less than 10 μg/kg bw of TCDD clearly induced cleft palate in C57BL/6 mice, 20 μg/kg bw of TCDD was necessitated to induce cleft palate in ddY mice. The fetuses of ddY mice were affected when 20 and 40 μg/kg bw of TCDD were administered on GD 12.5, indicating a "window effect" of TCDD on the induction of cleft palate; the incidence rate were 9.52% and 4.48%, respectively. At the concentration of 80 μg/kg bw TCDD, the cleft palate was induced in the fetuses administered on GD10.5, 11.5 and 12.5, of which the incidence rates were 6.9, 10 and 18.6%, respectively. In the ddY mouse, GD12 was the most sensitive gestation day for the induction of cleft palate when TCDD was administered per oral.

Gross and histological morphology

The cleft palates induced in the fetuses of C57BL/6 and ddY mice treated with by TCDD were typical in their morphology, having normal sized palatal shelves in a vertical position (Figure 1). Two palatal shelves failed to meet and fuse each other, resulting in a wide gap between them (Figure 1, 2). Histologically, the cleft was lined by nasal epithelial cells, medial epithelial cells of two opposing prominences, and then connected with squamous epithelial cells of oral cavity (Figure 2).

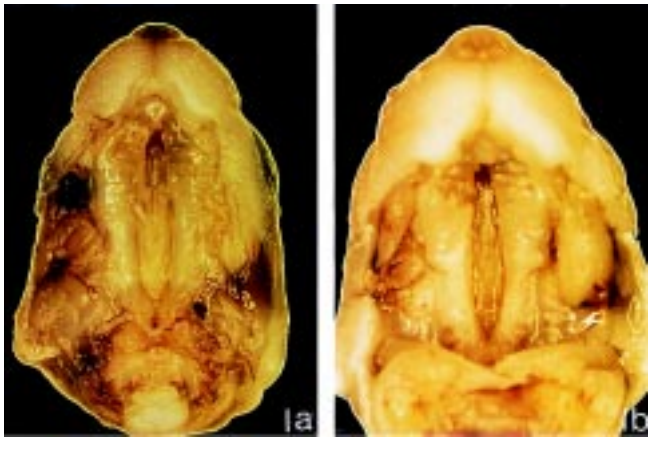

Fig. 1. The cleft palates induced in the fetuses of C57BL/6 (a) and ddY mice (b) treated with TCDD during pregnancy. Note the normal sized palatal shelves in a vertical position with a wide gap between the shelves.

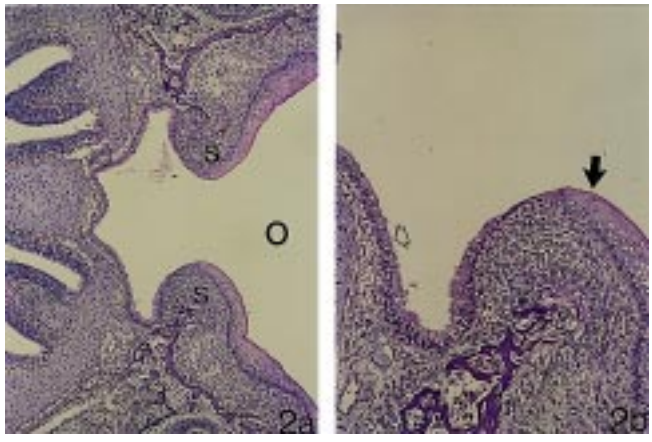

Fig. 2. Histological findings of cleft palate induced by TCDD. Two palatal shelves (S) fail to meet and fuse each other (a). Figure 2b is a high magnification of Figure 2a. Note cilliated columnar nasal epithelial cells (open arrow) which continue to flattened epithelial cells of two opposing prominences and squamous epithelial cells (arrow) of oral cavity (b). H&E, Magnification; a) × 50, b) × 100.

Discussion

It has been well documented that the induction of cleft palate is a toxic effect of TCDD on fetal development [1-5, 7-10, 21, 24, 25, 28]. The normal development of palate is completed by a growth of opposing palatal shelves and their fusion through the programmed cell death of medial edge epithelial cells [12]. Therefore, cleft palate can be induced by inhibiting the growth of medial epithelial cells or by interfering with a fusion between two palatal shelves. The cleft palate induced by TCDD is considered to result from the poor development of palatal shelves [28] or an altered differentiation of medial cells to interfere with the programmed cell death [2, 4, 25].

Our study confirmed that TCDD is a teratogen to induce cleft palate and has a "window effect" at low dosages for the induction of cleft palate. Morphologically, the cleft palates induced by TCDD in C57BL/6 and ddY mice were typically composed of normal sized palatal shelves in a vertical position, resulting from the failure of fusion between two opposing palatal shelves (Figure 1, 2). The incidence was the most sensitive when TCDD was treated around GD12 in both C57BL/6 and ddY mice. In C57BL/6 mice, the cleft palate was, at the concentration of 40 μg/kg bw, clearly induced when TCDD was subcutaneously treated only on GD 12.5; the incidence was 25 % (Table 1). When TCDD was orally administered, the incidence of cleft palate was also limited on GD10.5 ñ GD12.5 at the concentration of 10 μg/kg bw, indicating that the incidence of cleft palate is the most sensitive when TCDD is treated around GD12 (Table 3). The dose-increase to 20 μg/kg bw

Table 3. Fetal mortality and incidence of cleft palate in C57BL/6 mice following oral treatment of TCDD during pregnancy

Group (μg/kg)	GD (day)	GD 8.5	GD 9.5	GD 10.5	GD 11.5	GD 12.5	GD 13.5
0	No. of pregnant mother	3	5	4	4	3	2
	No. of fetus	25	34	28	30	25	16
	No. of early died fetus (%)[a]	1(4.0)	1(2.94)	0	2(6.67)	2(8.0)	1(6.25)
	No. of late died fetus (%)[a]	0	0	1(3.57)	0	1(4.0)	0
	No. of fetus with CP (%)[b]	0	0	0	0	0	0
20	No. of mother	3	5	3	4	2	3
	No. of fetus	27	44	24	35	18	27
	No. of early died fetus (%)	1(3.70)	1(2.27)	0	3(8.57)	0	0
	No. of late died fetus (%)	0	0	0	0	0	0
	No. of fetus with CP (%)	0	0	1(4.17)	12(37.5)*	5(27.8)*	0
40	No. of mother	3	4	5	4	5	4
	No. of fetus	22	35	43	40	46	38
	No. of early died fetus (%)	1(4.55)	2(5.71)	0	3(7.5)	2(4.35)	4(10.5)
	No. of late died fetus (%)	1(4.55)	3(8.57)	1(2.33)	2(5.0)	0	0
	No. of fetus with CP (%)	4(20.0)	8(26.7)	29(69.1)	33(94.3)**	43(97.7)**	12(35.3)
80	No. of mother	4	2	4	5	5	5
	No. of fetus	29	19	37	47	46	45
	No. of early died fetus (%)	0	1	0	2(4.26)	0	2(4.44)
	No. of late died fetus (%)	9(31.0)	1(5.26)	2(5.41)	7(14.9)	1(2.17)	0
	No. of fetus with CP (%)	20(100)**	16(100)**	34(97.1)	37(97.4)	45(100)**	22(51.2)*

[aa] % of affected fetuses / total fetuses

[b] % of affected fetuses / total fetuses

* $p<0.05$ vs control

** $p<0.01$ vs control

Table 4. Fetal mortality and incidence of cleft palate in ddY mice following oral treatment of TCDD during pregnancy

Group (μg/kg)	GD (day)	GD 8.5	GD 9.5	GD 10.5	GD 11.5	GD 12.5	GD 13.5
0	No. of pregnant mother	4	2	5	4	3	3
	No. of fetus	48	25	65	52	41	38
	No. of early died fetus (%)[a]	3 (6.25)	0	1 (1.54)	2 (3.85)	0	2 (5.26)
	No. of late died fetus (%)[a]	1 (2.08)	1 (3.45)	1 (1.54)	1 (1.96)	1 (2.44)	0
	No. of fetus with CP (%)[b]	0	0	0	0	0	0
20	No. of mother	3	2	5	5	3	2
	No. of fetus	38	27	69	63	42	25
	No. of early died fetus (%)	0	0	3 (4.35)	0	0	0
	No. of late died fetus (%)	0	2 (7.41)	1 (1.45)	0	0	2
	No. of fetus with CP (%)	0	0	0	0	4 (9.52)*	0
40	No. of mother	3	3	5	4	5	4
	No. of fetus	33	31	62	62	67	47
	No. of early died fetus (%)	2 (6.06)	0	0	3 (4.84)	3 (4.48)	2 (4.26)
	No. of late died fetus (%)	1 (3.03)	1 (3.23)	1 (1.61)	0	1 (1.49)	0
	No. of fetus with CP (%)	0	0	0	0	3 (4.48)*	0
80	No. of mother	4	4	4	5	5	5
	No. of fetus	46	51	42	63	61	59
	No. of early died fetus (%)	0	0	0	1 (1.59)	0	12 (20.3)
	No. of late died fetus (%)	1 (2.17)	1 (1.96)	13 (31.0)	2 (3.18)	2 (3.28)	1 (1.69)
	No. of fetus with CP (%)	0	0	2 (6.90)	6 (10.0)*	11 (18.6)*	0

[a] % of affected fetuses / total fetuses

[b] % of affected fetuses / total fetuses

* $p<0.05$ vs control

induced cleft palate on a wide range of gestation day (GD8.5–GD13.4), but the incidence was significantly high on GD11.5 and GD12.5 (Table 3). The "window effect" of TCDD for the induction of cleft palate was also observed in ddY mice at the concentration of 20 and 40 μg/kg bw as cleft palate was clearly induced when TCDD was orally administered only on GD 12.5 (Table 4). The incidence of cleft palate in TCDD-exposed embryos of C57BL/6 mice was in close agreement with that of the previous studies [7, 9].

In our study, it was found that ddY mice were very resistant to the fetal mortality and the induction of cleft palate following TCDD treatment. In fetal mortality, when TCDD was orally administered, the effects of TCDD appeared at 40 μg/kg bw in C57BL/7 mice (Table 3), while at 80 μg/kg bw of TCDD in ddY mice (Table 4). In the induction of cleft palate, when TCDD is injected subcutaneously on GD12.5, 80 μg/kg bw of TCDD failed to induce cleft palate in ddY mice (Table 2), which was comparable to 27.3 % incidence of C57BL/6 mice (Table 1). The resistance of ddY mice to the induction of cleft palate was also found when TCDD was orally administered. The oral treatment of 40 μg/kg bw of TCDD (a dose enough to affect all of fetuses in C57BL/6 mouse) to ddY mice on GD12.5 respectively induced cleft palate in only 18.6 % of fetuses (Table 4). The strain difference in our study might be due to a difference in the expression of AhR in the craniofacial tissue between the two mouse strains, since AhR mediates the induction of cleft palate by TCDD and its level may determine the sensitivity of animals. C57BL/6 mice highly sensitive to TCDD-induced cleft palate have been known to have high-affinity AhR in craniofacial tissues, while DBA/2J mice, TCDD non-responsive mice, have low-affinity AhR [23, 27]. AKR/J mice are also known to be a relatively insensitive to the induction of cleft palate by TCDD, which is also assumed to be due to the low-affinity AhR of the strain [25]. Therefore, in our study, the low sensitivity of ddY mice to the induction of cleft palate by TCDD may be explained on the basis of the previous studies even if there is no report regarding to the expression of AhR in the craniofacial tissue of ddY mice.

C57BL/6 and ddY mice were used to elucidate the mechanism through which PCTs induce cleft palate [17]. The previous study suggested that the cleft palate induced by PCTs be related with the up-regulation of corticosterone following PCTs treatment [17]. Nevertheless, the mechanism through which PCTs induce cleft palate is still unclear. In our study, the sensitivity of C57BL/6 and ddY mice to the TCDD-induced cleft palate was opposite to that of them to PCTs-induced cleft palate, indicating that the mechanism to induce cleft palate may be different between TCDD and PCTs. In addition, the increase of corticosterone level in plasma to have been observed after PCTs treatment in Kaneko`s study was not noted after TCDD treatment in our study (data not shown). It is also still unknown whether or not the toxicity of PCTs, like TCDD, is mediated by AhR.

Glucocorticoids (GC) are also teratogenic and induce cleft palate at pharmacological doses [11, 15, 16, 24]. GC and TCDD are known to give rise to their effects through binding the respective receptors, GR and AhR [24]. It is still unclear whether there is any interaction between GR and AhR during the normal development of palate or in the incidence of cleft palate. However, Abbott et al.'s studies had shown there may be a cross-regulation of GR and AhR, since the synergistic interaction between TCDD and hydrocortisone for the induction of cleft palate was found [1, 5]. According to their studies, TCDD treatment on GD14 induced up-regulation of GR and down-regulation of AhR, while the hydrocortisone exposure elevated the level of AhR and decreased the expression of GR. The treatment of both (TCDD + hydrocortisone) induced an increase of both receptors, followed by a synergistic increase of the incidence of cleft palate. The altered regulation of these receptors is followed by the altered expression of some growth factors [1, 3], resulting in altered differentiation and proliferation of palatal epithelial cells. The mechanism of interaction cycle between GR and AhR is still speculative.

In summary, the present study showed that ddY mice, a susceptible strain to PCTs-induced cleft palate, were very resistant to the induction of cleft palate by TCDD, suggesting that the mechanisms through which TCDD and PCTs induce cleft palate may be different. In addition, we confirmed a "window effect" of TCDD for the induction of cleft palate in ddY mice.

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