Biota Vol. 16 (2): 200−205, Juni 2011
ISSN 0853-8670

Critical Periods for TCDD-Induced Cleft Palate in Mice (*Mus musculus* L.)

Kerentanan Palatogenesis Mencit (*Mus musculus* L.) terhadap Induksi Cleft Palate TCDD

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Abstract

An experiment was conducted in order to test the susceptibility of mice palatogenesis to the effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD). The experiments were designed according to Completely Randomized Design with a 4 X 3 factorial experiment. Forty-eight pregnant mice were given TCDD at a dose of 0 (control), 5, 10 or 20 µg / kg bw on gestation days (GD) 9–10, 11–12, or 13–14. TCDD was solubilized in DMSO, diluted in sesame oil to appropriate volume and administered to mice orally by gavage; control group received the vehicle only (98.5% sesame oil + 1.5% DMSO). On GD 18 mice were anesthetized and then killed by cervical dislocation. Fetuses with cleft palate (cp) were calculated (%), degree of palatal closure were scored, and the feature of cleft palate were observed in 6 µm craniofacial microsections. Result showed, TCDD treatment on GD 9 to 12 induced cp; the highest induction was on GD 9–10 treatment. TCDD dose of 10 or 20 µg / kg bw when given on GD 9–10 or on GD 11–12 induced cp in more than 90% of the offspring. The percentages of cp were still high when the treatments were given on GD 11–12, especially with dose of 20 µg / kg bw (87.3%). The lowest dose of TCDD (5 µg / kg bw) induced cp which was dominated by narrow-gap feature, indicating that fusion stage was disturbed; dose of 10 or 20 µg / kg bw induced cp with intermediate-gap or wide-gap feature, suggested that there has been a disruption in initiation or elevation stage. In conclusion, all stages of palatogenesis were susceptible to TCDD effects, but the most susceptible was fusion stage.

Key words: TCDD, palatogenesis, *cleft palate*

Abstrak

Telah dilakukan percobaan untuk menentukan tahapan palatogenesis pada mencit (*Mus musculus* L.) yang rentan terhadap efek polutan 2,3,7,8-Tetraklorodibenzo-*p*-dioksin (TCDD). Percobaan dirancang mengikuti Rancangan Acak Lengkap dengan pola faktorial (4X3). Empat puluh delapan ekor mencit bunting dicekok TCDD dengan dosis 0 (kontrol), 5, 10, atau 20 µg/kg bb. Perlakuan diberikan pada hari kebuntingan (Hk) 9−10, 11−12, atau 13−14. Mencit kontrol dicekok pelarut saja (98,5% minyak wijen + 1,5% DMSO). Pada Hk 18 mencit dibius lalu dibunuh dengan teknik *cervical dislocation*, persentase fetus *cleft palate* (cp) dihitung, derajat penutupan palatum diberi skor, preparat dengan ketebalan 6 µm dibuat, dan mikrostruktur kraniofasial diamati. Hasil menunjukkan, pemberian TCDD antara hari ke 9 dan 12 menginduksi cacat cp, dengan kecenderungan hasil tertinggi pada pemberian Hk 9–10. Perlakuan TCDD dosis 10 atau 20 µg/kg bb pada Hk 9–10 menghasilkan fetus cacat cp >90%. Persentase fetus cp tetap tinggi pada pemberian Hk 11–12, khususnya pada kelompok dosis 20 µg/kg bb (87,3%). TCDD dosis terendah (5 µg/kg bb) menginduksi cp dominan bercelah sempit, menunjukkan adanya hambatan pada tahap fusi. Dosis 10 dan 20 µg/kg bb menginduksi cp bercelah sedang atau lebar, mengisyaratkan terjadi hambatan pada tahap inisiasi atau elevasi. Disimpulkan, seluruh tahapan palatogenesis rentan terhadap efek TCDD, namun tahap paling rentan adalah tahap fusi palatum.

Kata kunci: TCDD, palatogenesis, *cleft palate*

Diterima: 05 Agustus 2010, disetujui: 25 Januari 2011

Introduction

Mechanism of secondary palate development, or palatogenesis, has been described elsewhere. In brief, palatogenesis consisted of three stages: initiation, elevation, and fusion. In the first stage, the secondary palate arises as bilateral outgrowths from the maxillary processes. They form primordia of the palatal shelves which start to grow downward laterally to the tongue (vertical growth). At the second stage, palatal shelves growth is reoriented and begin to elevate to a horizontal position above the tongue. Their subsequent horizontal growth resulting in the approximation of both opposing palatal shelves. Palatogenesis culminates in the third stage (fusion) when medial edge epithelium (MEE) from both shelves forming an epithelial seam which is later replaced by mesenchymal cells to form the definitive palate (Ferguson, 1988; Kerrigan *et al*., 2000; Meng *et al*., 2009). Cleft palate (cp) is congenital fissure in the roof of the mouth, resulting from incomplete fusion of palatal shelves during embryonic development. Cleft palate may be resulted from disturbance of processes at any stage of palatogenesis (Ferguson, 1988).

Xenobiotic 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) belongs to dioxin group, a group of unwanted byproduct chemicals, generated by many industries and combustion processes. Dioxin is highly toxic, chemically persistent, environmentally mobile, and readily bioaccumulated through food-web (Willis, 2000). The chemical has been known to have teratogenic effect in various animals, including induced cp in mice (Lilienfield and Gallo, 1989). However, the critical periods for induction of cp by TCDD remain obscure.

It has been suggested that TCDD inhibit programmed cell death in fusion stage, resulting in cp with narrow fissure (Abbott and Birnbaum, 1991). However, it was established later that, there was no such inhibition related to TCDD (Takagi *et al*., 2000). Mechanism of cleft formation was then proposed as due to cell kinetic problems leading to poor development of palatal shelves and resulting in cp with small and diminutive palatal shelves (Takagi *et al*., 2000). Again, the last mechanism was argued by Yamada *et al*., (2006), by showing that in TCDD treatment, palatal shelves grew to the same extend as in control group, and completely contacted one each other, but fail to fuse. It was therefore suggested, some disturbances might have occurred during adhesion of opposing palatal shelves (Yamada *et al*., 2006). On the other hand, there was also reported that TCDD delay the process of shelves elevation, resulting in cleft palate with both palatal shelves remain in vertical position (Yoon *et al*., 2000). In this study, we tested a hypothesis that critical periods for induction of cp by TCDD occured in all the stages of palatogenesis, which depend on doses of treatment and time of TCDD administration.

Materials and Methods

The chemical 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Neosyn Lab., USA, in >98% purity. TCDD solution was prepared by dissolving it in Dimethylsulfoxyde (DMSO). The solution was then diluted with sesame oil to adjust the concentration. Pregnant Swiss mice were obtained by overnight mating, and the present of vaginal plug was considered as gestation day 0 (GD 0). The dams were maintained during pregnancy in animal house facilities (*Unit Pengembangan Hewan Percobaan* UGM) with Japfa Comfeed par G pellet and *ad libitum* tap water.

Fourty eight pregnant mice were arranged according to completely randomized design with a factorial experiment. The first factor was TCDD doses, and the second factor was time of TCDD administration. The mice were devided into 4 groups based on the level of doses. Group I (control) received oral administration of vehicle only (1.5% DMSO + 85.5% sesame oil). Group II, received TCDD 5 μg/kg bw, group III received 10 μg/kg bw, and group IV received 20 μg/kg bw. Each group were further devided into 3 subgroups of 4 mice based on time of administration. Subgroup A were given TCDD on gestation day (GD) 9−10, subgroup B on GD 11−12, and subgroup C on GD 13−14. Doses selected to be tested has been reported to cause cleft palate,

but here they were evaluated in combination with time of treatment corresponding to palatogenesis stage. On GD 18, all of the subgroup animals were anesthetized with ether and then killed by cervical dislocation. Fetuses were removed from the uterus. Incidence of cp were determined as percentage of fetuses with cp from all alive fetuses. A cut was made through the mouth of embryo to facilitate macroscopic examination of the palate. Degree of palatal closure were scored according to Abbott *et al*., (1994). Palatal closure were classified into four categories (score): 1) wide fissure, opposing palatal shelves were wide apart, separated by a distance ≥palatal shelves width, 2) moderate fissure, palatal shelves were close together, separated by a distance <palatal shelves width, 3) narrow fissure, palatal shelves were in contact but not fusing; 4) no fissure, complete palatal closure. Two fetuses from each dam were selected for microstructure preparation. They were decapitated and processed for craniofacial coronal sections of 6 µm thickness by routine microsection techniques (Drury and Wallington, 1976).

Incidence of cp was analyzed by ANOVA, followed by Scheffe's test for post hoc analyses. A probability of less than 5% was considered significant (Sokal and Rholf, 1986).

Results and Discussion

Incidence of Cleft Palate

The results showed TCDD clearly induced cp. Statistical analysis by two way ANOVA revealed that interaction between TCDD dose and time of administration was significant ($P<0.05$), meaning that the incidence of cp was depended on dose of TCDD and time of administration. By one way ANOVA, the effect of every dose and time of administration levels were analized separately. Finally, post hoc analysis by Scheffe's test revealed the difference between treatment combination (Table 1).

The combination of high TCDD dose and early time of administration (20 µg/kg bw given on GD 9−10) produced cp in almost all of the offsprings, (32 fetuses with cp out of 33 alive fetuses) (Table 1). This combination, along with two other combination group (10 µg/kg bw given on GD 9−10, and 20 µg/kg bw given on GD 11−12), produced the highest incidence of cp. There was a tendency that the earlier TCDD given caused the higher incidence of cp (Figure 1). TCDD have almost totally disappeared its potency to induce cp when it was given on GD 13−14. Only one fetus out of 96 alive fetuses developed cp when TCDD was given on GD 13−14 (Table 1).

Scores of Palatal Closure

Degrees of completeness of palate development varied in fetuses with cp. Distribution of palatal closure score based on TCDD dose and time of administration is presented in Figure 2. The figure gave interesting detail of characteristic of cp induced by TCDD. In lower dose (5 µg/kg bw, group II) given on any gestation day (on GD 9−10 or GD 11−12) the palatal shelves was dominated by narrow fissure (score 3) (in total, 38 out of 39 fetuses). On the other hand, score 1 only appeared in fetuses of group III and IV (Table 1, Figure 2).

Cleft Palate Features

Gross and microscopic observation of craniofacial showed, there were various features of cp induced by TCDD (Figure 3). The features ranging from cp with very wide fissure, moderate fissure, to narrow fissure. The narrow fissure varied from unilateral to bilateral cp. In all fetuses, palatal shelves have positioned horizontally above the tongue. There were no cp found with palatal shelves remain in vertical position.

In Figure 1, it is clearly showed that cp only developed when TCDD were given on GD 9–10 or on GD 11–12, but not on GD 13–14. Lack of cp-inducing effect of TCDD when given on GD 13–14 consistent with the previous report. According to Birnbaum (1998) dioxin treatment on GD 13 was much less effective at inducing clefting, and from GD 14 on, cleft palate could not be induced by prenatal dioxin exposure. This appear to be due to the fact that palatal fusion occurs on GD 14. Treatment at the time or later cannot block the fusion process.

The cleft features with wide and moderate fissure (score 1 and 2; Figure 2) indicated that TCDD disturbed specific developmental processes leading to decrease of palatal growth. Since growth of a developing structure depended on the rate of celluler proliferation and the production of extracelluler matrix substance (Kerrigan *et al*., 2000), both might be involved in TCDD-decreasing palatal shelves growth, but the mechanism need further research.

Our result showed that TCDD did not delay selves elevation, as revealed by the fact that there was no cp with palatal shelves remain in vertical orientation found in this study. This finding does not agree with previous study which reported TCDD delay shelves elevation in C75BL/6 and ddY mice which resulting in cp with vertical type (Yoon *et al*., 2000). The different final features of cp may be resulted from the different strain of animal used in experimentation, and the different in doses of TCDD and time of administration (Altshuler *et al*., 2003; Rogers and Kavlock, 2007).

Table 1. Effects of TCDD doses and time of its administration on incidency of cleft palate and score of palatal closure in mice.

TCDD Doses (µg/kg bw)	Time of Administration (GD)	Number of			Incidency of cp (%)
		Dams	Alive Fetuses	Fetus With cp	
0	9−10	4	38	0	0 ^{a}
	11−12	4	37	0	0 ^{a}
	13−14	4	37	0	0 ^{a}
5	9−10	4	38	24	60.3 ^{bcd}
	11−12	4	39	15	39.3 ^{b}
	13−14	4	23	0	0 ^{a}
10	9−10	4	37	35	94.8 ^{e}
	11−12	4	38	20	52.8 ^{bc}
	13−14	4	41	0	0 ^{a}
20	9−10	4	33	32	98.3 ^{e}
	11−12	4	33	28	87.3 ^{cde}
	13−14	4	33	1	2.5 ^{a}

Means followed by different superscripts differ significantly (P<0.05), by Sceheffe's test.

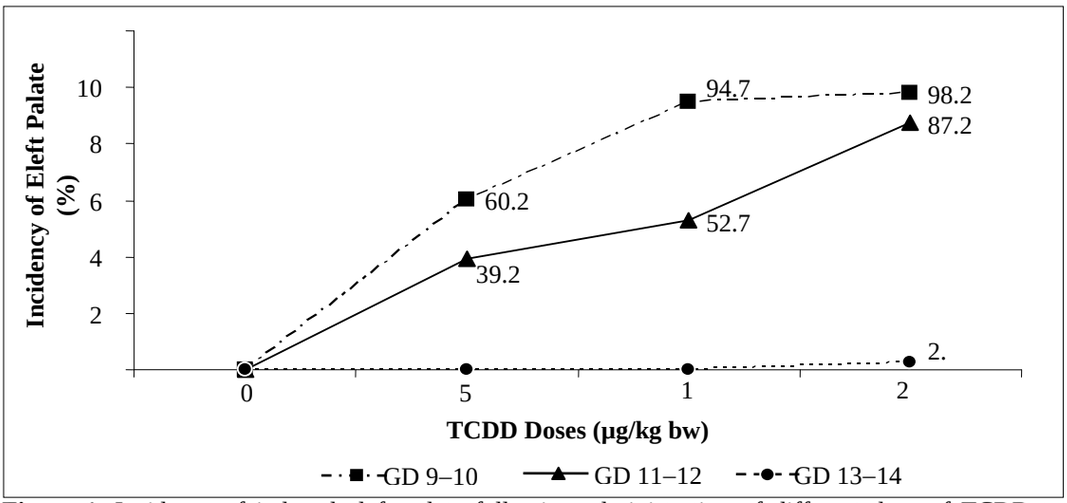

Figure 1. Incidence of induced cleft palate following administration of different dose of TCDD to pregnant mice at various times during gestation. TCDD treatment between GD 9 and 12 induced cleft palate, with a tendency toward maximal response on gestation day 9–10. More than 90% of fetuses had cleft palates when 10 or 20 µg/kg bw TCDD were administered on GD 9–10. The responses remained high on GD 11–12 of treatments, especially in 20 µg/kg bw group (87.25%). Exposure on gestation day 13–14 almost did not induce cleft palate.

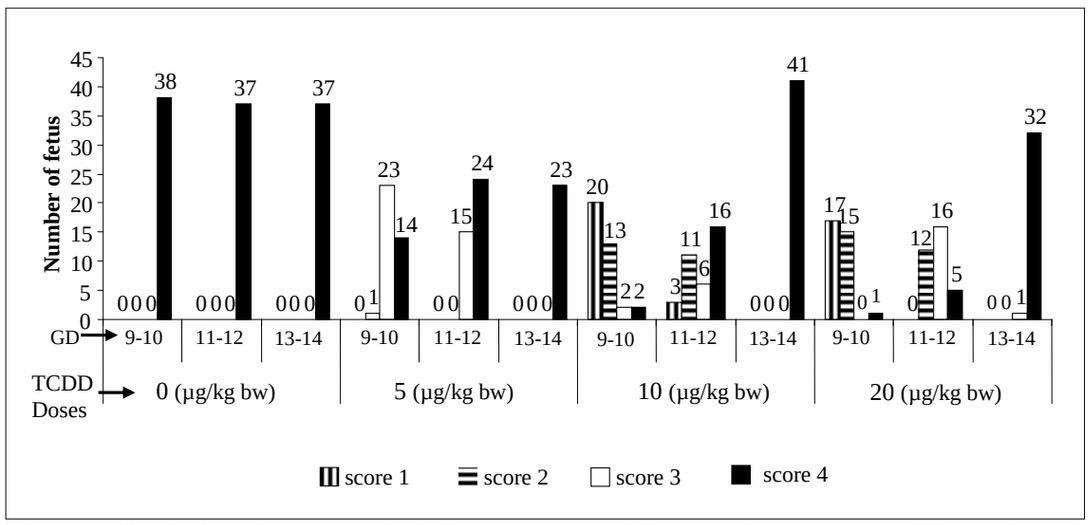

Figure 2. Distribution of score of palatal closure in fetuses following administration of different dose of TCDD to pregnant mice at 3 different gestation days. Cleft palate induced by low dose of TCDD (5 µg/kg bw) were dominated by cleft palate with narrow fissure (score =3), while the higher dose (10 and 20 µg/kg bw) resulted in moderate or wide fissure (score =2, or =1). GD: Time of TCDD administration (gestation day); Doses: TCDD doses (µg/kg bw). Number at the end of bar =number of fetuses.

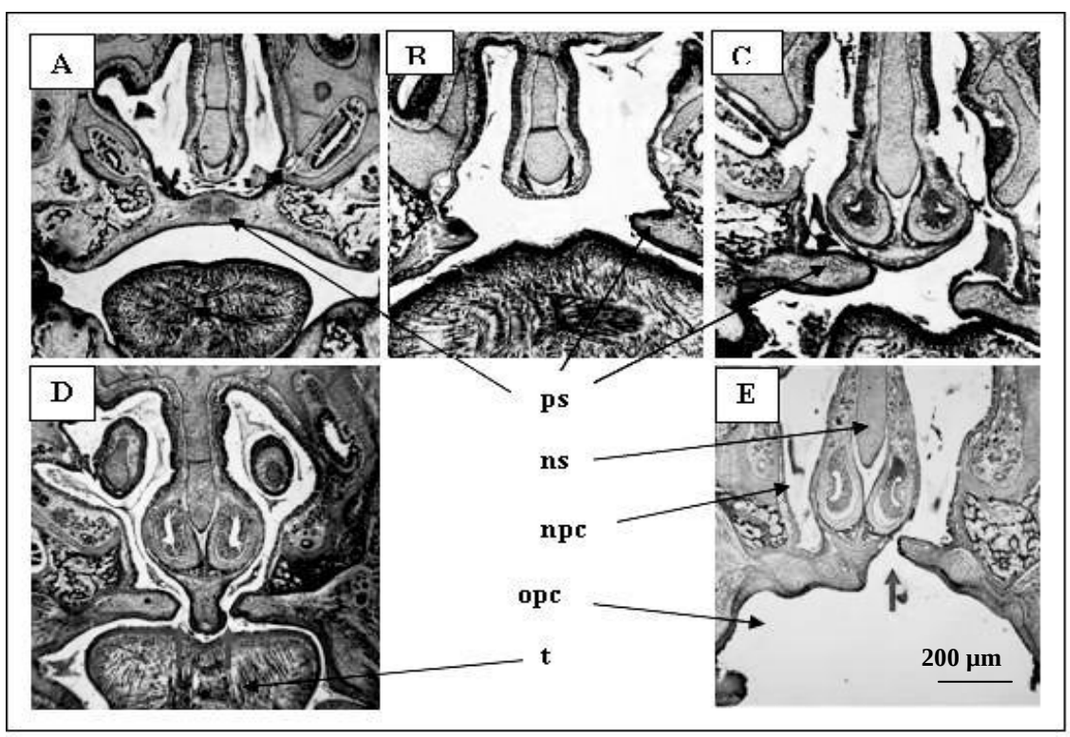

Figure 3. Representative photomicrograph of mouse fetuses craniofacial microstructure affected by TCDD. A. Normal palate. B-E. Fetuses with cleft palate. B. Cleft palate with wide fissure. C. Cleft palate with moderate fissure. D and E. Cleft palate with narrow fissure. Sample taken from GD 18 fetuses; Fissure or cleft in the mouth roof indicated by bold arrow (in B, C, and E), and by white arrows in D; ps: palatal shelf; ns: nasal septum; npc: nasopharyngeal cavity; opc: oropharyngeal cavity; t: tongue.

The fact that TCDD given on GD 13−14 did not induce cp, it does not mean that the fusion stage is unsensitive to TCDD effect. It is evident that a lot of cp fetuses found in this study developed the feature of narrow fissure (63 out of 155 clefted fetuses), which is the characteristic of cp resulted from disruption of fusion stage (score = 3) (Abbott dan Birnbaum, 1991). Palatal fusion occur in mouse on GD 14, but TCDD given around the time (Gd 13−14) fail to induce cp, it seems that preparation for fusion which sensitive to TCDD effects occur before GD 13.

An interesting result derived from all dams treated with lower dose TCDD (5 µg/kg bw). The treatment induce cp with exclusively narrow fissure type (score =3; Figure 2). The dose of 5 µg/kg bw seems to be not enough to affect palatal shelves growth in initiation and elevation stage, but enough to affect fusion stage as indicated by the feature of cp resulted which were predominantly with a narrow fissure feature. It is probably that palatal fusion stage is the most vulnerable stage of palatogenesis to TCDD effect.

Conclusion

TCDD induced cleft palate by disturbing palatogenesis at any stage. The most critical period is probably the last stage (fusion), but in order to affect the stage, TCDD must be given before GD 13. Shelves elevation *per se* is not affected by TCDD.

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