3. Results

3.1. Pregnancy outcomes

TCDD administration apparently had no effect on maternal health. Pregnancy outcomes are summarized in Table 1. Abortions, stillbirths, and early postnatal deaths occurred at fairly high frequencies in the TCDD-treated groups as well as the control group. The postnatal mortality rate of the offspring was higher in the 300-ng/kg group (50%) than in the control group (28%), but the difference was not statistically significant ($P>0.1$). In an attempt to increase the number of surviving offspring in the 300-ng/kg group, we added nine dams to the group approximately 2 years after the initiation of the experiment. However, only two surviving offspring were added, due to a high incidence of abortions. There were no significant differences in the average length of gestation and average birth weight among the three groups.

Table 1
Pregnancy outcome and postnatal mortality

Dose of TCDD	No. of dams	No. of abortions	No. of stillbirths	No. of live births	No. of early postnatal deaths[a]	Gestation length (days)	Birth weight (g)
Control	23	2	3	18	5	161.8±7.8	426.1±58.6
30 ng/kg	20	0	5	15	3	163.8±5.9	426.8±56.9
300 ng/kg	20	2	2	16	8	164.9±9.7	402.7±62.1
300 ng/kg[b]	9	5	1	3	1	165.0±3.0	466.0±87.1

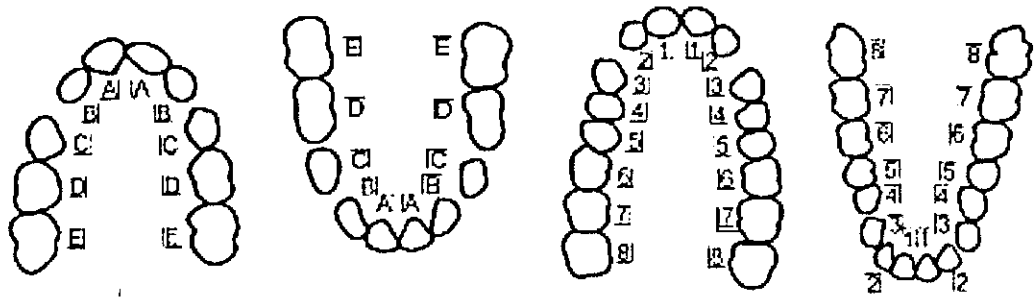
[a] Death by PND 100.
[b] Additional group.

3.2. Dental findings

3.2.1. Dentition in normal rhesus monkeys

The number and types of teeth of the rhesus monkey are similar to those of humans. The number of deciduous and permanent teeth are 20 and 32, respectively. Figure 1 illustrates outlines of these teeth and the code for designation of each tooth used in Tables 3 and 4. Neonatal monkeys usually have no erupted teeth. The central incisors erupt during the first postnatal month. The approximate ages of eruption of deciduous teeth are 1 month for the lateral

incisors, 2 months for the canines, 2.5 months for the first molars, 5 months for the second molars in the lower jaw, and 7 months for the second molars in the upper jaw. Those of permanent teeth are 2.5 years for the central incisors, 2.7 years for the lateral incisors, 3.5 years for the canines and the first premolars, 2.7 years for the second premolars, 1.5 years for the first molars, 3.5 years for the second molars, and 5.5 years for the third molars [11]. During this study we found that there were fairly large interindividual variations for the age of eruption of teeth.



Childhood upper jaw Childhood lower jaw Adult upper jaw Adult lower jaw

Fig.1 Diagram illustrating outlines of rhesus teeth seen from the occlusal plane and the code for designation used in Tables 3 and 4.
A-E: Deciduous teeth A: Central incisor B: Lateral incisor C: Canine D: First molar
E: Second molar
1-8: Permanent teeth 1: Central incisor 2: Lateral incisor 3: Canine 4: First premolar
5: Second premolar 6: First molar 7: Second molar 8: Third molar
⌋: Upper right ⌊: Upper left ⌉: Lower right ⌈: Lowere left

3.2.2. Dental findings in stillborn offspring and those that died postnatally

The incidences of tooth abnormalities are given in Table 2. During the early stage of this study, some carcasses from stillbirths and early postnatal deaths were discarded inadvertently; therefore the numbers of specimens in Table 2 are smaller than the total numbers of stillbirths and early postnatal deaths in Table 1. Stillborn fetuses from the control group had no erupted teeth in either the upper or lower jaw (Fig. 2A, E). However, conventional radiographs clearly revealed the presence of 20 well-formed deciduous teeth (Fig. 3A, E). Each tooth could be identified by its characteristic shape and size.

Dental examination of the dead offspring revealed tooth abnormalities only in the 300-ng/kg group. Three of the five animals had tooth abnormalities such as precocious eruption, dysplasia, incomplete calcification, and missing teeth. Although the incidence of tooth abnormalities in the 300-ng/kg group was high (60%), it did not differ significantly from the control incidence (0%; $P>0.1$), perhaps because of the small sample size. Descriptions of offspring with

tooth abnormalities follow, and representative macroscopic photographs and conventional radiographs are shown in Figures 2 and 3, respectively. Abnormal findings are summarized in Table 3.

Table 2
Incidence of tooth abnormalities

Group	Stillbirths and early postnatal deaths			Surviving offspring		
	No. of offspring	No. of offspring with tooth abnormalities (%)		No. of offspring	No. of offspring with tooth abnormalities (%)	
Control	4	0	(0)	13	0	(0)
30 ng/kg	5	0	(0)	12	0	(0)
300 ng/kg	5	3	(60)	8	6	(75*)
300 ng/kg[a]	1	0	(0)	2	1	(50)

[a] Additional group.
* Significantly different from the control group ($P<0.01$).

Table 3
Tooth abnormalities detected in stillbirths and early postnatal deaths in the 300-ng/kg group

Offspring No.	Sex	Death categories	Age[a]	Abnormal findings
34[c]	♂	Abortion	GD 128	-[b]
37[d]	♂	Stillbirth	GD 164	A|AD $\overline{A\|A}$ premature eruption, dysplasia |B missing
40[d]	♀	Early postnatal death	PND 26	|D premature eruption, incomplete calcification A|A missing
43[c]	♂	Stillbirth	GD 176	-
57[c]	♂	Early postnatal death	PND 1	BA|ABD $\overline{A\|A}$ premature eruption, incomplete calcification
103[c]	♀	Stillbirth	GD 173	-

[a] GD: gestation days; PND: postnatal days.
[b] No abnormalities were detected.
[c,d,c] Total dose of TCDD administered to the dams: [c]345, [d]360, [c]375 ng/kg.

Offspring No. 37 was stillborn on GD 164. The deciduous upper central incisors and left first molar had erupted precociously (Fig. 2B). The erupted teeth were irregular in shapes and apparently were destroyed. X-ray examination revealed incomplete calcification in the erupted teeth, and the deciduous upper left lateral incisor was missing (Fig. 3B). The deciduous lower central incisors also had erupted precociously; these teeth were dark brown

(Fig. 2F), and their calcification seemed slightly retarded (Fig. 3F) as compared with that of a control animal stillborn at an earlier gestational age (Fig. 3E, No.10: stillborn on GD 146).

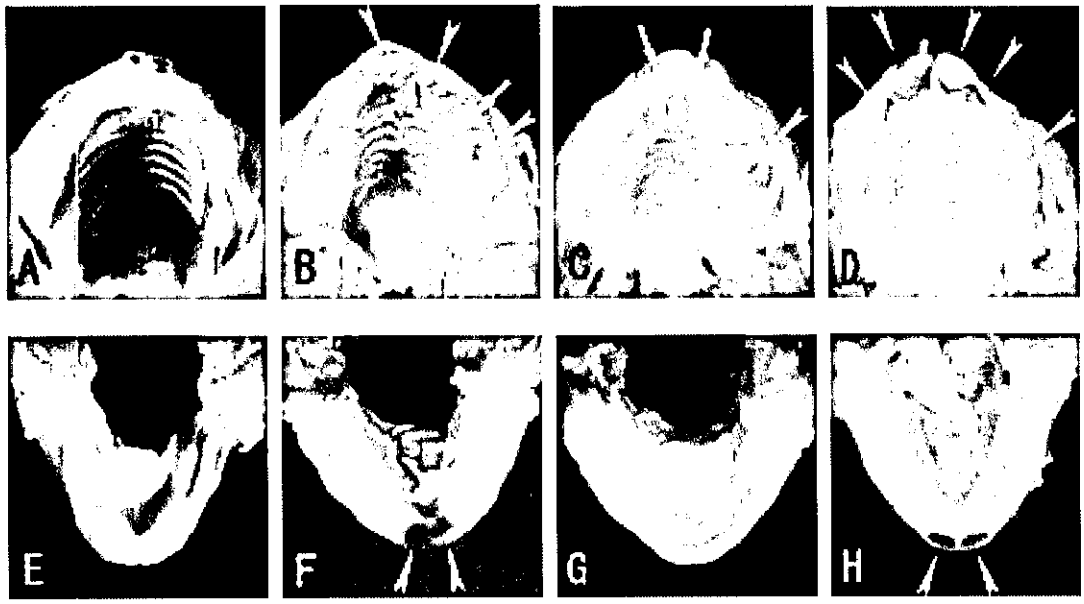


Fig. 2. Macroscopic photographs of jaws from a control offspring (A, E) and from offspring in the 300-ng/kg group with tooth abnormalities (B-D, F-H) which were stillborn or died early postnatally. Upper jaws (A-D) and lower jaws (E-H). Arrowheads indicate precocious eruption. Arrows point the location of missing teeth detected by X-ray. Offspring numbers and ages: A, E: No. 10, GD 146; B, F: No. 37, GD 164; C, G: No. 40, PND 26; D, H: No. 57, PND 1.

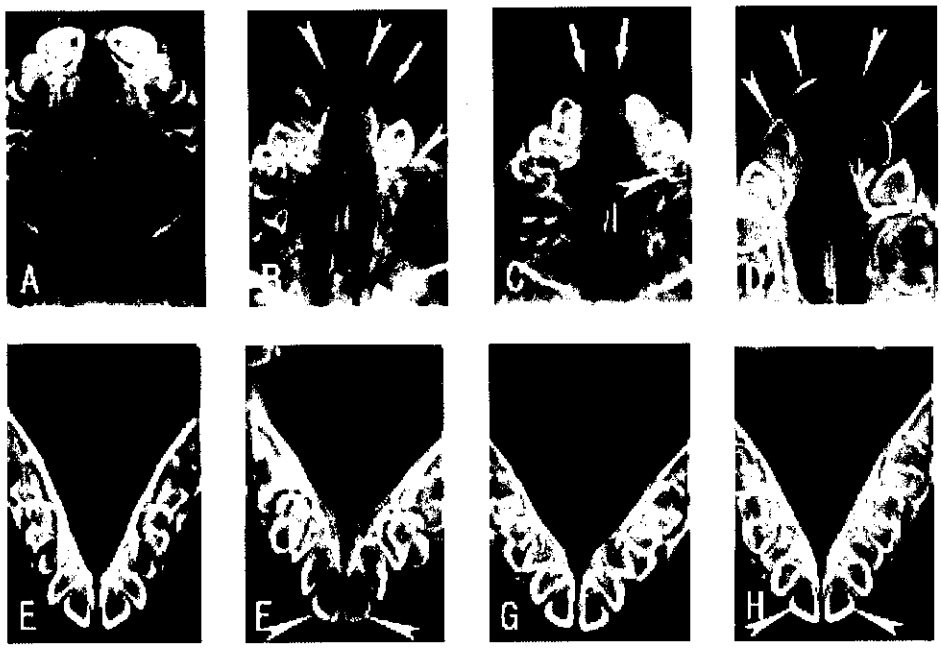


Fig. 3. Conventional radiographs of jaws shown in Fig. 2. Radiographs are arranged corresponding to Fig. 1. Upper jaws (A-D) and lower jaws (E-H). Arrowheads and arrows indicate precocious eruption and missing teeth, respectively. Offspring numbers: A, E: No. 10; B, F: No. 37; C, G: No. 40; D, H: No. 57.

Offspring No. 40 died postnatally on PND 26. The deciduous upper left first molar had erupted precociously (Fig. 2C). The four cusps were discernible macroscopically but were unclear in the radiograph (Fig. 3C), indicating retarded calcification of the tooth. X-ray examination revealed that both the deciduous upper central incisors were missing. A slight deviation of the anterior nasal septum to the left was noted (Fig. 3C). The lower teeth were still in the gum, and no abnormality was detected radiographically (Fig. 3G).

Offspring No. 57 died when a neonate, on PND 1. The bilateral deciduous upper central and lateral incisors, upper left first molar (Fig. 2D) , and bilateral lower central incisors (Fig. 2H) had erupted precociously. The lower incisors were dark brown. X-ray examination revealed retarded calcification in these precociously erupted teeth (Fig.3D, H).

3.2.3. Dental findings in surviving offspring

The incidences of tooth abnormalities in the surviving offspring are given in Table 2. The incidence in the 300-ng/kg group (total, 70%) was significantly higher than that in the control group (0%; $P<0.01$). Representative macroscopic and radiographic photographs are shown in Figures 4 and. 5, respectively. Abnormal findings are summarized in Table 4.

3.2.3.1. Offspring observed between approximately PND 800 and PND 1400

In the vehicle-treated group, offspring were at the stage of losing the deciduous teeth during the period of PND 800 to PND 1400. In the majority of animals, the permanent central and lateral incisors and the first molars had erupted. By conventional radiography, all the permanent teeth except for the third molars were detectable. Descriptions of a control animal and the monkeys from the 300-ng/kg group with tooth abnormalities follow.

Offspring No. 1 is a control animal. Figure 4A shows the central upper and lower jaws on PND 1438. The permanent central and lateral incisors as well as canines had erupted. Figure 5A is a radiograph of the anterior upper jaw taken on PND 1049. The midline is approximated by the left border of the picture. The deciduous central incisor had been lost, and the permanent central incisor had erupted. The deciduous lateral incisor still remained but was being pushed up by the growing permanent incisor. The permanent canine was discernible deep to the long root of the deciduous canine on the distal side of the root of the permanent lateral incisor. The canine could be easily identified by the pointed shape of the crown. Figure 5B shows the upper left molars radiographed on PND 1438. The deciduous first molar had been lost, and the first premolar had erupted. The crown of the deciduous second molar remained posterior to the permanent first premolar, and it was being pushed up by the permanent second premolar. The permanent first and second molars had erupted, but the third molar could not be seen clearly. Figure 5C shows the lower right lateral incisor, canine, and molars on PND 1438. The lateral incisor and canine were permanent teeth, but the deciduous first and second molars still remained, being pushed up by the growing permanent first and second premolars. The permanent first molar had erupted.

Offspring No. 31 through No. 66 are members of the 300-ng/kg group.

Offspring No. 31 was observed macroscopically on PND 1430. It was found that both the upper permanent lateral incisors were missing (Fig. 4B, arrows). The upper left second premolar had erupted but its crown was small and cone-shaped (Fig. 4C, arrowhead). These defects were confirmed by radiography (Fig. 5D, arrow; Fig. 5, E arrowhead). Radiographs taken on PND 1430 showed that the upper left deciduous first molar remained, and no permanent first premolar was found between the roots of the deciduous first molar (Fig. 5E, arrow); therefore a missing permanent first premolar was diagnosed. Similar findings from the upper right side led to diagnosis of missing first and second premolars on this side. In the lower jaw, the right second premolar had erupted, but its crown was on the

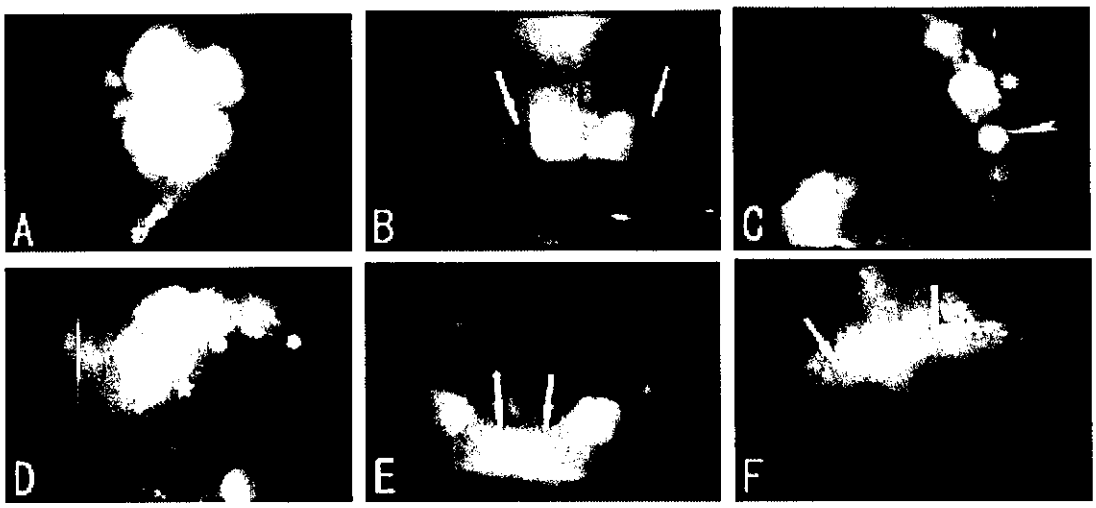


Fig. 4 Macroscopic photographs of surviving offspring in the control group (A) and 300-ng/kg group with tooth abnormalities (B-F). Arrows: missing; arrowhead: cone-shaped; star: maldirected; asterisks: remaining deciduous teeth. Offspring numbers and ages: A: No. 1, PND 1438; B, C: No. 31, PND 1430; D: No. 44, PND 1415; E: No. 66; PND 1338; F: No. 106, PND 688. A: upper and lower incisors; B: upper incisors; C: upper left molars; D: lower right molars; E: lower incisors; F: upper incisors.

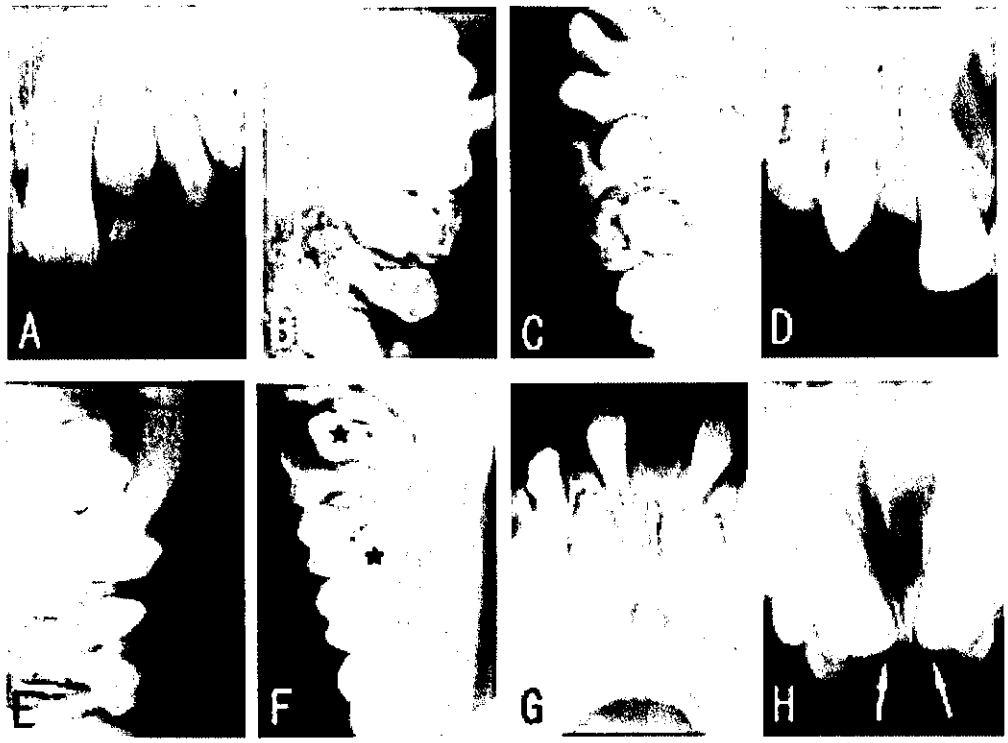


Fig. 5. Conventional radiographs of surviving offspring in the control group (A-C) and 300-ng/kg group with tooth abnormalities (D-H). Arrows: missing; arrowhead: cone-shaped; stars: maldirected. Offspring numbers and ages: A: No. 1, PND 1049; B, C: No. 1, PND 1438; D, E: No. 31, PND 1430; F: No. 44, PND 1415; G: No. 66, PND 1338; H: No. 106, PND 688. A: upper left incisors and canine; B: upper left molars; C: lower right molars; D: upper right incisor and canine; E: upper left molars; F: lower left molars; G: lower incisors; H: upper incisors

Table 4

Tooth abnormalities detected in surviving offspring in the 300-ng/kg group

Offspring No.	Sex	Age[a] at observation	Abnormal findings
31[c]	♀	941, 1041, 1122, 1430	542|24 missing, |5 cone-shped
33[d]	♂	960, 1060, 1449	-[b]
35[d]	♀	936, 1036, 1425	-
39[c]	♂	921, 1021, 1102, 1410	542|245 missing
42[d]	♀	926, 1026, 1415	5|5 missingh, |4 cone-shaped
44[d]	♂	926, 1026, 1415	$\overline{54}\|45$ maldirected
60[d]	♂	899, 999, 1080, 1388	542 |245 $\overline{5}\|\overline{5}$ missing
66[c]	♂	849, 949, 1030, 1338	52|2 $\overline{1}\|\overline{1}$ missing, |45 cone-shaped, maldirected, $\overline{54}\|45$ maldirected
106[d]	♀	198, 299, 380, 688	A|A 4|24 missing
109[d]	♀	189, 290, 679	-

[a] Postnatal days.

[b] No abnormalities were detected.

[c,d] Total dose of TCDD administered to the dams: [c]420, [d]405 ng/kg.

lingual side of the alveolar gum. Radiographically, the axis of the second premolar was inclined in a lingual and distal direction.

Offspring No. 39: was examined radiographically on PND 1410. The upper permanent lateral incisors and the first and second premolars were missing bilaterally.

Offspring No. 42 had an upper left first premolar that was cone-shaped, and the upper second premolars were missing bilaterally, according to observation on PND 1415.

Offspring No. 44 had a lower right first premolar that was inclined in a lingual and mesial direction, and it had erupted on the lingual side of the alveolar gum (PND 1415; Fig. 4D, star). Because of this maldirection, the deciduous first molar still remained. Similarly, the lower right second premolar was maldirected, and the deciduous first and second molar also remained (Fig. 4D, asterisks). The lower left first premolar had not erupted, but a radiograph sowed that the first and second premolar were maldirected (Fig. 5F, stars), and the deciduous first and second molars remained.

Offspring No. 60 was evaluated macroscopially and radiographically on PND 1388. These studies indicated that both lateral incisors and both first and second premolars were missing from the upper jaw. In addition, both second premolars were missing from the lower jaw.

Offspring No. 66 had an upper jaw from which the bilateral permanent lateral incisors and the right second premolars were missing, and the left first and second premolars were cone-shaped. In the lower jaw, the permanent central incisors were found to be absent on PND 1338 (Fig. 4E, arrows). The remaining permanent incisors were close to the canines, and there was a wide space between the two incisors (Fig. 5G, arrow), indicating that the incisors were lateral ones. The upper left first and second premolars had been erupted by PND 1338, but were cone-shaped, and maldirected. The lower first and second premolars were also maldirected bilaterally.

3.2.3. 2. Offspring observed between approximately PND 200 and PND 700

The two surviving offspring that were added to the 300-ng/kg group were approximately PND 200 at the time of their first radiographic examination. They were followed until approximately PND 700. Only one of these animals, **Offspring No. 106,** had obvious tooth abnormalities. This animal had a wide space between the small incisors in the upper jaw; this defect first was observed on PND 198, and was confirmed on PND 688 (Fig. 4F, arrows). Radiographs taken on PND 688 showed the growing permanent central incisors and a wide median gap between the remaining deciduous teeth (Fig 5H, arrows), indicating that the deciduous central incisors were missing. In addition, the upper bilateral first premolars and the left permanent lateral incisor were missing.

4. Discussion

The results of the present study clearly showed that prenatal and lactational exposure to TCDD with an initial dose of 300 ng/kg and a maintenance dose of 15 ng/kg affected tooth development in rhesus monkeys. The exposure began on GD 20, when the rhesus embryo is at the stage of primitive streak formation, corresponding to Carnegie stage 8 in the human embryo [12], and no tooth germs are present. In humans, the dental lamina, the earliest indication of teeth, appears by the sixth week of development [13]. The human embryo at 6 weeks after fertilization (Carnegie stage 17) corresponds to the rhesus embryo at 5 weeks after fertilization [14]. The human permanent tooth bud first appears around 10 weeks after fertilization, which corresponds to approximately 8 weeks after fertilization in the rhesus.

According to our observations, the 20 deciduous teeth had been well shaped by the time of delivery in the control offspring. Although the last maintenance injection of TCDD was done on PND 90, the offspring were considered to be exposed to TCDD via milk until weaning, approximately 1 year after birth. Even after weaning, TCDD that had accumulated in the various tissues gradually was released into the blood and could have affected developing permanent teeth. Hence, it is reasonable to assume that deciduous as well as permanent teeth were exposed to TCDD throughout the critical period of development and that the observed tooth abnormalities were associated with TCDD exposure.

Unfortunately, some of the carcasses from the stillbirths and early postnatal deaths were discarded and therefore unavailable for dental examination. However, all the dental abnormalities we identified were noted after the disposal, and we feel that no bias was introduced into the sampling of the specimens. The incidences of tooth abnormalities among stillbirths and early postnatal deaths did not differ significantly between the control (0%) and 300-ng/kg (60%) groups, probably because of the small sample size. However, the difference among the surviving offspring is statistically significant, and we therefore reasonably consider that all the observed tooth abnormalities are due to TCDD exposure.

Developmental studies with TCDD in the rhesus monkey have been performed for more than 25 years and by several groups [15-19]. The main finding in these studies was abortions, and tooth abnormalities were not reported. However, one dioxin-related compounds, 3,4,5,3',4',5'-hexachlorinated biphenyl (Aloclor 1242) has been reported to affect tooth development in rhesus monkeys [20]. These animals had cystic periodontal lesions around the unerupted molars 13 months after consumption of food containing 400 ppm Aloclor 1242 for 40 days, suggesting that dioxins might affect developing teeth in primates.

TCDD affects tooth development in rodents. A single oral dose of 1 μg/kg to pregnant rats on GD 15 disturbed postnatal molar development in the offspring [21]. Lactational exposure through maternal intraperitoneal administration of TCDD to rats at a dose level of 1000 μg/kg on PND 1 also affected molar development in the offspring [22,23]. In addition, growing incisors in rats were sensitive to continuous exposure to TCDD for 20 weeks beginning from 10 weeks of age.[24].

Human epidemiological studies have been conducted to examine possible association between dioxin exposure and tooth abnormalities. In Finland, 102 6- to 7-year-old children who were breast-fed for an average of 10.5 months were studied. Milk samples were collected when the children were 4 weeks old, and the concentrations of dioxins and furans were determined. The total exposure to dioxins was calculated from the concentrations in milk and the duration of breast feeding. The frequency and severity of hypomineralization of teeth correlated with the total exposure [25].

Follow-up studies after accidental exposure to dioxins also have indicated that the teeth are targets of developmental toxicity of these toxicants. High frequencies of delayed eruption and missing permanent teeth occurred among children with fetal Yusho or Yuchen (oil disease), which occurred in 1968 in Japan and in 1979 in Taiwan after ingestion of rice oil contaminated with PCBs and PCDFs [6,7]. In addition, examination of 48 people exposed to dioxins because of the notorious accident in Seveso, Italy, in 1976 revealed a high incidence of developmental defects of enamel and missing permanent teeth [5]. These subjects had been younger than 9.5 years at the time of the accident and were examined for tooth abnormalities 25 years afterward. Plasma collected in 1976 had TCDD concentrations that ranged from 23 to 26,000 ng/kg in serum lipid. Subjects with higher serum TCDD levels had developmental dental defects more often than those with lower TCDD levels.

In the present study, we found positional differences among teeth as manifestations of the sensitivity to the developmental toxicity of TCDD. Even before eruption, each tooth can be easily identified in light of the position of the canine, which is large and has a characteristically pointed crown. The canines were not affected in any of our monkeys; the vulnerable teeth were the central and lateral incisors, deciduous first molars, and the first and second premolars. In the patients with Yusho, the most frequent missing tooth was the lower premolar, followed by the lower lateral incisor [6]. In humans the lateral incisor and the second premolar are considered to have a regressing tendency in the process of evolution [26], and these teeth are missing relatively frequently in the general population. This intrinsic regressive tendency might be exacerbated by exogenous toxicants, resulting in positional differences in sensitivity.

It is well known that interactions between the ectoderm covering the first branchial arch and the mesenchyme derived from the neural crest are important in tooth morphogenesis. Several signal molecules and their receptors have been identified [27]. TCDD is a potent modulator of epithelial cell growth and differentiation [28], and most of its toxic effects are mediated by the aryl hydrocarbon receptor (AhR) [29]. For example, cleft palate induction by TCDD was completely abolished in AhR knockout mice [30]. In mouse tooth buds, AhR is expressed in secretory odontoblasts and ameloblasts [23], suggesting the pathway via AhR as a mediator of dental toxicity of TCDD. One candidate for the pathway of TCDD action on tooth morphogenesis involves epidermal growth factor (EGF) and the EGF receptor (EGFR). TCDD added to cultured embryonic mandibular molar tooth germs induced depolarization of ameloblasts and disturbed morphogenesis [31]. EGF added to the TCDD-containing medium suppressed the adverse effects of TCDD. The effect of TCDD was less dramatic on tooth germs from EGFR knockout mice [31]. Although no study has assayed expression of EGF or EGFR during tooth morphogenesis in rhesus embryos, these findings suggest that the EGF-EGFR signaling system may work in tooth development in the rhesus monkey as well as the mouse and that disturbance of this system by TCDD results in dysmorphogenesis of rhesus teeth.

In addition to altered epithelial-mesenchymal interaction, excessive apoptosis may be involved in the pathogenesis of tooth defects. TCDD added to organ culture of mouse molar tooth germs did not affect cell proliferation but increased apoptosis in the epithelium, resulting in defective molar [32]. In the process of cleft palate induction in mice by TCDD, excessive apoptosis was observed in the epithelium covering the palatal processes and in the palatal mesenchyme [33]. It is plausible that apoptosis induced by TCDD played a role in induction of tooth defects in the present experiment. Cleft palate was not detected in the present study. Probably the dose levels were too low to induce cleft palate in the rhesus monkey. In the sensitive C57 BL strain of mice the LOAEL level for induction of cleft palate was reported to be 3000 ng/kg/day by oral administration during the period of organogenesis [34]. Detailed examinations of possible target organs of developmental toxicity of TCDD including the urinary, reproductive, and immune systems are in progress.

Our examination of the surviving offspring until the age of approximately 4 years revealed tooth defects only in the 300-ng/kg group. By macroscopic observation with the digital camera we could not detect mineralization defects reported in humans [5,25]. Because the permanent molars are still developing in 4-year-old rhesus monkeys, detailed further observation may reveal some subtle abnormalities such as enamel defects in the offspring currently diagnosed as normal in the 30- and 300-ng/kg groups. Blood samples taken from pregnant and lactational mothers and milk samples await analyses for TCDD concentrations. Although the dosing schedule in the present study was set to keep the body burden at 30 or 300 ng/kg, the actual maternal body burden should be assessed after the autopsy of the

mothers and analyses for TCDD, because the TCDD half-life in rhesus monkeys has shown fairly large interindividual variations [10]. Plasma samples taken at intervals from the offspring are also waiting for TCDD analyses. Assuming that the actual body burden was not much different from the programmed one, the LOAEL body burden for the developmental toxicity of TCDD in rhesus monkeys is considered to be somewhere between 30 and 300 ng/kg and is probably on the order of 86 ng/kg, the value used to set the current TDI in Japan. In 2002, a panel of experts surveyed various data in the literature and concluded that no urgent change was necessary in the current TDI of 4 pgTEQ/kg/day [35]. The results of our present study support this conclusion. However, we should wait to draw a definite conclusion until the measurement of the actual body burden of the dams and detailed examinations of various organs of the offspring.

Acknowledgement

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Title:

Contamination of Dioxins in Free Ranging and Breeding Monkeys in Japan and Relationship Analysis between Limb Malformations and Administration with 2, 3,7,8- Tetrachlorodibenzo-p-dioxin (TCDD) on Macaque Monkeys

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Key words: dioxins, TCDD, monkey, limb malformation, contamination, blood

(Abstract)

In this study, we investigate to be fine that relations are presence or not between dioxins and the limb malformations to be born in wild in macaque monkeys. The contamination of dioxins in the monkey blood between the malformed and the normal was measured using mass-spectrometer. The dioxin isomers except 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, and 1,2,3,7,8,9-HxCDF were detected in the bloods of the two groups of monkeys. TEQs from the monkeys with the abnormal were lower than TEQs from the monkeys with the normal. The TEQs of the monkeys with abnormal limbs, ID 2 and ID 3ID in wild monkey are much lower than that of the monkeys ID 1 with normal limb in the same group of wild monkey. And also pregnant monkeys were exposed with 2,3,7,8-Tetrachlorodibenzo-p-dioxin from the day 20 of gestation to the day 90 after birth and the limb was examined on neonates born from the exposed pregnant. These 42 neonates exposed with TCDD had observed with normal limbs. Finally present investigations resulted to indicate that dioxins including TCDD had no or more weak effect than that of thalidomide on the limb malformations at the range of 30-300 ng TEQ /kg of body weight to macaque monkeys.

1. Introduction

Among the environmental pollutants, dioxin is highly toxic even at low levels on immune (Pohjanvirta and Tsuomisto, 1994), reproductive (Birnbaum, 1994) and developmental systems (Birnbaum, 1995) in human, wildlife, and experimental animals.

Comparing the many reports on the effects of dioxins in rodents (Abraham et al., 1989, Nagao et al., 1996, Michalek et al., 1996, Gray et al., 1997), not much is studied on the effects in non-human primates. In the point of view among species differences, the studies using primates are needed to estimate dioxin affects on human health. For experimental animals, macaque monkey has advantages for human models because of the most resemblance in physiological properties.

In the wild life of the macaque monkeys, the limb malformations of monkeys in Japan have been reported since 1957 (Itani and Mizuhara, 1957, Yoshihiro et al., 1979) and that is still to be seen in monkeys in Japan. Their chromosomal abnormalities are excluded from the cause of the limb malformations (Minezawa et al., 1990). Therefore the environmental pollutant chemicals are most suspected the influence on the malformations. The Dioxin contaminations in monkey in Japan have reported by the survey of the Ministry of Environment in Japan (the Ministry of Environment in Japan, 1999), but it has not been available information about the contamination of dioxins in wild living monkeys with limb malformations.

The development of limb buds is established as stage 12 for human embryo and comes apparent at day 28 of gestation in the embryo of the macaque monkeys (Hendrickx and Peterson, 1997). Limb malformations are exerted by the administration of chemicals such as thalidomide to pregnant monkeys before the day 28 of gestation (Hendrickx and Peterson, 1997). The macaque monkeys are an animal model to be inducted the limb defects in human thalidomide syndrome that have not been produced in rodent embryo because of species differences between rodent and primates including humans.

Considering the advantage of monkeys for human models, we have been studying the effects of administration of TCDD on the tissue functions and the organ development in the pregnant monkeys (Kubota et al, 2000, Kubota et al, 2001, Asaoka et al, 2003, Asaoka et al, 2004, Yasuda et al, 2005).

In this study, we investigate to be fine that relations are presence or not between dioxins and the limb malformations to be born in wild in macaque monkeys. The contamination of dioxins in the monkey blood between the malformed and the normal was measured using mass-spectrometer. And also pregnant monkeys were exposed with 2,3,7,8-Tetrachlorodibenzo-p-dioxin from the day 20 of gestation to the day 90 after birth and the limb was examined on neonates born from the exposed pregnant. Finally present investigations resulted to indicate that dioxins including TCDD had no or more weak effect than that of thalidomide on the limb malformations at the range of 30-300 ng TEQ /kg of body weight to macaque monkeys.

2. Methods and Materials

2.1. Chemicals.

2, 3,7,8-TCDD dissolved in toluene and DMSO (1:2, V/V) was purchased from Daiichi Pure Chemicals Co., Ltd. Tokyo, Japan.

2.2. Measurement of dioxin isomers in blood and TEQ calculation

The isomers of dioxins in 10 ml of blood samples from the monkeys was measured in Towa Kagaku Co., Ltd., Hiroshima, Japan, using a high resolution mass spectrometer by the methods of the provisional manual for analyzing the dioxin in blood by the Ministry of Health, Labour and Welfare (22 Dec, 2000). TEQ of the monkey blood was calculated by the amount of dioxin isomers using WHO-TEF (WHO, 1998). Isomers that were detected at levels below the lower limit of determination were assigned as not detected (ND), and concentrations below the lower limit of determination were converted to TEQ values equivalent to zero values.

2.3. Blood samples

Blood samples were collected from two groups of Japanese monkeys living in different districts on February-March 2003. One is provisional group of free ranging wild monkeys including limb malformations at Arashiyama, Kyoto Japan and the other is breeding monkeys at Primate Research Institute, Kyoto University, Aichi, Japan. The birth at Primate Research Institute has not been found with limb malformation since the establishment of breeding facilities on 1980. Both monkeys were captured and treated according to the animal control program and the guide for the care and use of laboratory primates. Four blood samples were obtained from each group. Two abnormal monkeys with limb malformations were from the group at Arashiyama. The blood samples were collected after anaesthetization with Ketaral and kept in freezing until used. The ages of the monkeys tested were conformed by the records of birth.

2.4. Pregnant monkeys and TCDD administration

Rhesus monkeys purchased from China National Scientific Instruments & Materials Import/Export Corporation (Beijing, China). The monkeys (6-9 years old and 4.5-6.5 kg in body weight) were kept in Shin Nippon Biomedical Laboratories, Ltd, Kagoshima, Japan. The breeding conditions were described previously (Ihara et al., 1999). The monkeys were mated, and the pregnancies were administered 2, 3,7,8-TCDD (30-300 ng / kg of body weight) via subcutaneous on day 20 of their gestation. Every 30 days interval, 5% of the initial dose of TCDD was given to the pregnancies until day 90 after birth for maintaining the body burden. Controls were given the solvent only.

3. Results and Discussion

3.1. Blood analysis

The monkeys captured at the wild group have two monkeys with limb malformations in total four monkeys. The abnormality was types of split hands and lack of arms shown in figure 1. This type of limb abnormality categorized in absence deformities, which are the most frequently observed in the limb malformations in Japan (Yoshihiro et al., 1979). The breeding monkeys at Inuyama had normal llimbs as same shown in figure 1.

The contamination of dioxin in the blood from wild and breeding monkeys in Japan was measured using mass-spectrometer as summarized in Table I. The dioxin isomers except 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, and 1,2,3,7,8,9-HxCDF were detected in the bloods of the two groups of monkeys. Amount of each isomer in the monkey blood was no difference among the limb properties, different districts, and sexes. By sum of dioxin isomers measured, the amounts of coplanar polychlorobiphenyl,

polychrolodibenzo-p-dioxine, and polychlorinated dibenzofran were detected in the range of 830-31,000 pg/ g lipid, 22-82 pg/ g lipid, and 4.1-67 pg/ g lipid, respectively, in the bloods of monkeys at both places.

Although lack of blood samples and abnormal monkeys, the survey reports almost same range of the dioxins and the presence of 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, and 1,2,3,7,8,9-HxCDF in wild monkeys by the Ministry of Environment in Japan (The Ministry of Environment in Japan, 1999). The amount of dioxins in the blood of monkeys with abnormal limbs was within the same ranges of the amount of dioxins in that of normal monkeys by the present data and the reported by the Ministry.

TEQ was calculated by the amount of the isomers using WHO-TEF as summarized in Table II. The TEQ levels in the monkey blood were also no difference among the limb properties, different districts, and sexes. The Total TEQ calculated by the amount of all isomers in monkey blood with limb malformations was also in the same ranges of that with normal limbs, 5.5-17 pg TEQ / g lipid, and 3.6-34 pg TEQ/ g lipid, respectively.

The TEQs of each individual was pointed against their birth year as shown figure 2. The TEQ from the monkey with the abnormal was pointed in the range of TEQ from the normal breeding monkeys. TEQs from the monkeys with the abnormal were lower than TEQs from the monkeys with the normal. The TEQs of the monkeys with abnormal limbs, ID 2 and ID 3ID in wild monkey are much lower than that of the monkeys ID 1 with normal limb in the same group of wild monkey. This shows the no relations between limb abnormality and the effects of dioxins incorporated to the monkeys.

3.2. Exposed analysis

Twenty neonates were born from 20 pregnancies administered with 30 ng / kg of body weight of TCDD, the 22 neonates were born from 29 pregnancies administered with 300 ng / kg of body weight of TCDD, and 21 neonates were born from 23 pregnancies of control. These 42 neonates exposed with TCDD had observed with normal limbs and the control neonates were also born with normal limbs. The 9 abortions could not be detected.

The TCDD administration was started to monkey pregnancies from the day 20 of gestation that is pre-periods before the day 28 of gestation that begins the limbs formation in monkey embryo (Hendrickx and Peterson, 1997). Present study was enough at the stage of limb formation to examine the possibility of the effect of TCDD on limb malformations because the defects in thalidomide syndrome are inducted by chemical administration at day 24-30 of gestation in macaque monkeys of rhesus and Japanese monkeys (Wilson and Gavan, 1967, Tamimura, 1972).

Finally present investigations resulted to indicate that dioxins including TCDD had no or more weak effect than that of thalidomide on the limb malformations at the range of 30-300 ng TEQ /kg of body weight to macaque monkeys. The limb malformations in wild may be occurring with other environmental material(s) or their complex and/or with TCDD in the environments surrounding the monkeys. The abnormal birth in wild monkeys are serious for humans because the physiological resemblance between humans and monkeys. Further studies are necessary to clarify the relationships between environmental pollutants and the limb malformation to be born in wildlife.