on infant health. Unfortunately, there is no generally accepted Test Guideline for newborn toxicity studies. Therefore, we established a new protocol (Koizumi *et al.*, 2001) in order to investigate the differences of susceptibility between the newborn and young rats. This protocol includes a detailed examination of physical development and sexual maturation, and a complete toxicity analysis after the 9-week recovery-maintenance period, the age of the young adult. Furthermore, a unique feature is that the same lot number of chemical and the same rat strain from the same supplier are used as in young rat studies.

Using this protocol, we have already tested 14 phenolic derivatives as a part of an existing chemical testing program of Japan in 1999. So far, we have reported three comparative analyses of 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol (Koizumi *et al.*, 2001, 2002a, 2002b; Yamamoto *et al.*, 2001; Takano *et al.*, 2001; Nishimura *et al.*, 2002). In these studies, for more precise / appropriate comparison, we estimated both the no observed effect levels (NOAELs) and unequivocally toxic levels, defined as doses inducing severe toxic signs including death or critical histological damage, based on the results of both the main studies and the dose-finding studies for each case. In consequence, it was concluded that the susceptibility of newborn rats to the toxicity of these chemicals ranged from 2 to 4 times that of young rats.

In the present study, we selected 3-methylphenol, widely known as m-cresol and used in synthetic resins, disinfectants and pharmaceutical raw materials (Chemical Products' Handbook, 2002). Several reviews on the toxicity of this chemical or cresols, including three isomers, have been published (ATSDR, 1991; EHC, 1995; IRIS, 1997). For 3-methylphenol, although various clinical signs, growth inhibition and some developmental effects have been reported (TRL, 1986; BRRC, 1988a, 1988b, 1989; MBA, 1988; NTP, 1992), there are no data to our knowledge on its direct effects in newborn animals. In this study, we estimated the NOAELs and unequivocally toxic levels of 3-methylphenol, and compared them between newborn and young rats employing the previously described protocol.

MATERIALS AND METHODS

Materials

3-Methylphenol (CAS No. 108-39-4, purity: 99.13%) was obtained from Honshu Chemical Industry Co., Ltd. (Wakayama, Japan), and dissolved in olive oil. The test solution was prepared at least once a week and kept cool and in the dark until dosing. The stability was confirmed to be at least 8 days under these conditions. All other reagents used in this study were specific purity grade.

Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at 20-26°C with a relative humidity of 45-65%, a ventilation rate of more than 10 times per hour, and a 12:12 hr light/dark cycle. In the 18-day main study of newborn rats, 21 pregnant rats (gestation day 15) were purchased and allowed to deliver spontaneously. Among all newborns separated from dams at postnatal day 3 (the date of birth was defined as postnatal day 0), 48 males and 48 females were selected by stratified random sampling based on the body weight and assigned to 4 dose groups, including controls. Twelve foster mothers suckled the 4 males and 4 females assigned to each group up to weaning on postnatal day 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group were individually maintained for 9 weeks. In the 28-day study of young rats, 4-week-old male and female rats were obtained and used at ages of 5 weeks after acclimation. All animals were allowed free access to basal diet (newborn rat study: LABO MR stock, Nihon Nosan Kogyo Inc., Yokohama, Japan; young rat study: CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water.

Study design (Time schedule as reported previously (Koizumi *et al.*, 2001))

1. 18-Day repeated dose study in newborn rats

1) Dose-finding study

Newborn rats (5/sex/dose) were administered the test substance at 0, 100, 300 or 1,000 mg/kg/day in olive oil by gastric intubation daily from postnatal days 4 to 21. They were examined for general behavior and body weights during the dosing period, and sacrificed at postnatal day 22 after overnight starvation, for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

2) Main study

Newborn rats (12/sex/dose) were administered 3-methylphenol at 0, 30, 100 or 300 mg/kg/day in olive oil by gastric intubation daily from postnatal days 4 to 21, based on results of the dose-finding study, and 6

males and 6 females in each group were sacrificed on postnatal day 22 after overnight starvation. Recovery-maintenance groups (rest of animals in all groups) were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age. General behavior was noted at least once a day for newborn rats (separated from each foster mother) and foster mothers. Body weight was measured at postnatal days 4, 7, 10, 13, 16, 19 and 21, and then at 7-day intervals, and food consumption (for 24 hr from the day before) at the same days after weaning. At postnatal day 20 for males and day 21 for females, gait condition, pupillary reflex, auricular reflex, corneal reflex, visual placing reflex, surface and mid-air righting reflexes, and ipsilateral flexor reflex were examined. Furthermore, fur appearance, incisor eruption and eye opening were observed in all animals from postnatal days 7, 9 and 11, respectively, and testes descent and vaginal opening were examined from postnatal days 17 and 29, respectively. Color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity and volume of the urine were examined only at the end of the recovery-maintenance period. Blood was collected from the abdominal aorta under ether anesthesia at sacrifice after overnight starvation for scheduled-sacrifice and recovery-maintenance groups. One part was treated with EDTA-2K or 3.8% sodium citrate and examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count, and leukocyte analysis percentage, as well as blood clotting parameters such as prothrombin time and activated thromboplastin time. Serum obtained from another portion of the blood was analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-GTP), lactate dehydrogenase, cholinesterase, calcium, inorganic phosphorus, sodium, potassium, chlorine). After gross examination of the body surface, orificial mucosa and internal organs of animals sacrificed by exsanguination following collection of blood, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, prostates, seminal vesicle, ovaries and uterus were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The trachea, stomach, intestine, pancreas, lymph glands, urinary bladder, spinal cord, bone marrow and sciatic nerve as well as the above organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopic examination. For other groups, the organs with macroscopically abnormal findings or in which dose-related effects were evident on microscopic examination for the highest dose group, were examined.

2. 28-Day repeated dose study in young rats

1) Dose-finding study (14-day study)

Five-week-old rats (5/sex/dose) were administered the test substance at 0, 125, 250, 500 or 1,000 mg/kg/day in olive oil by gastric intubation for 14 days. They were examined for general behavior, body weight and food consumption during dosing and sacrificed after overnight starvation following the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

2) Main study

Five-week-old rats were given the test substance in olive oil by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment. Referring to the results of the dose-finding study, 4 doses, including the control, were established (0, 100, 300, 1,000 mg/kg/day). Recovery groups (0, 1,000 mg/kg) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. The number of animals for each sex/dose was 7 for both scheduled-sacrifice and recovery cases. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Data were statistically analyzed as follows (Sakuma, 1977, 1981; Yamasaki *et al.*, 1981). Continuous data were analyzed by Bartlett's test for distribution. When homogeneity was recognized, one-way analysis of variance was performed. When a significant difference was observed, Dunnett's or Scheffe's tests

were conducted for comparisons between control and 3-methylphenol-treated groups. If not homogenous or in the case of quantitative urinalysis data, analysis was performed using the Kruskal-Wallis test. In consequence, if a significant difference was detected, Dunnett type, Scheffe type or Mann-Whitney's U tests (Mann and Whitney, 1947) were conducted. In the newborn rat study, categorical data for general appearance, reflex ontogeny, necropsy and histopathology were analyzed by Fisher's exact probability test. A probability less than 5% was considered statistically significant.

RESULTS

18-day study in newborn rats (including the dose-finding study)

In a dose-finding study at doses of 100, 300 and 1,000 mg/kg, all animals at 1,000 mg/kg died within two days after the first treatment (Table 1). These rats showed deep respiration, decrease in spontaneous activity and pale skin. At 300 mg/kg, deep respiration and tremors under contact stimulus were noted during the dosing period in all animals, but no deaths occurred. No clinical signs were observed at 100 mg/kg. Body weight gain was depressed in females at 300 mg/kg. Blood biochemical examination showed a slight increase in total bilirubin in both sexes receiving 300 mg/kg (males; 0.36 mg/dL, compared with 0.32 mg/dL in controls, females; 0.37 mg/dL, 0.32 mg/dL). Organ weights, shown in Table 2, demonstrated a significant increase in relative liver weight in males at 100 and 300 mg/kg, and in females at 300 mg/kg but not absolute liver weights. No dose-related changes in hematology or gross findings were observed. Based on these results, the clearly toxic dose of 300 mg/kg was selected as the top dose in the main study, and 30 and 100 mg/kg were derived by approximately one-third divisions.

In the main study, various toxic signs such as deep respiration, increase in motor activity and tremors under contact stimulus were noted during the dosing period in all animals receiving 300 mg/kg, but no deaths occurred (Table 1). With 100 mg/kg, although

Table 1. Clinical signs and mortality in 18-day studies of 3-methylphenol in newborn rats.

	Dose-finding study			Main study		
Dose (mg/kg)	100	300	1,000	30	100	300
Males						
No. of animals	5	5	5	12	12	12
No. of dead animals	–	–	5[a]	–	–	–
No. of animals with clinical signs						
Deep respiration	–	5	3	–	–	5
Increase in motor activity	–	–	–	–	–	12
Decrease in spontaneous activity	–	–	5	–	–	1
Hypersensitivity on handling	–	–	–	–	1[b]	7
Tremors under contact stimulus	–	5	–	–	3[c]	12
Pale skin	–	–	1	–	–	–
Females						
No. of animals	5	5	5	12	12	12
No. of dead animals	–	–	5[a]	–	–	–
No. of animals with clinical sign						
Deep respiration	–	5	2	–	–	3
Increase in motor activity	–	–	–	–	–	12
Decrease in spontaneous activity	–	–	5	–	–	1
Hypersensitivity on handling	–	–	–	–	–	10
Tremors under contact stimulus	–	5	–	–	–	12
Pale skin	–	–	2	–	–	–

–: No animals with clinical sign.

[a]: All animals died within 2 days after first treatment, [b]: Observed only at dosing day 18, [c]: Observed only at dosing day 4 in one rat and at dosing day 11 in another two.

no clinical signs were observed in the dose-finding study, three males showed tremors under contact stimulus only on single days, dosing days 4, 11 and 11, respectively, and another male showed hypersensitivity on handling on a single day, dosing day 18. No change in general behavior was observed at 30 mg/kg. Body weights of both sexes given 300 mg/kg were lowered during the dosing period, but at 100 and 30 mg/kg were comparable to control values (Fig.1). No definitive changes in developmental parameters, including sexual maturation, as well as reflex ontogeny were detected in any dose group. At the scheduled sacrifice, blood biochemical examination of the 300 mg/kg group showed increases in γ-GTP, total bilirubin and BUN in males (Table 3). Significant increase of relative liver weight but not absolute liver weight was noted in both sexes given this highest dose (Table 2). In addition, there was a decrease in absolute brain weight in both sexes, but this change was not noted in the dose-finding study. On histopathological examination, basophilic tubules in kidneys showed a tendency to increase in 300 mg/kg males (slight and moderate changes in 2/6 and 3/6, respectively, compared with only slight change in 5~6/6 animals in other groups). After the recovery-maintenance period, there were no dose-related changes in body weight, blood biochemistry and histopathology, but low absolute brain weights remained in males (1.90 g, compared with 2.08 g in controls). No dose-related changes in food consumption, urinalysis, hematology and gross finding were observed throughout this study, including the recovery-maintenance period.

Since the hypersensitivity on handling and tremors under contact stimulus observed in a small number of males of the 100 mg/kg group were considered as dose-related adverse effects, the NOAEL in the main study was concluded to be 30 mg/kg/day. However, these clinical signs at 100 mg/kg were observed only on single days during the dosing period in the main study and not in the dose-finding study. Therefore, as for the unequivocally toxic level, 300 mg/kg/day was concluded to be appropriate because significant toxic effects in the central nervous system were observed at this dose, along with decrease in body weight gain.

28-day study in young rats (including the dose-finding study)

In the dose-finding study for 14 days at doses of 125, 250, 500 and 1,000 mg/kg, no deaths occurred at any dose (Table 4). Salivation, tremors and prone/lateral position were observed during the dosing period in both sexes at 1,000 mg/kg. Body weights and food consumption were lowered in males receiving 1,000 mg/kg. At 500 mg/kg and less, no changes in clinical signs, body weight and food consumption were observed. Blood biochemical examination showed increase in total cholesterol in females at 1,000 mg/kg

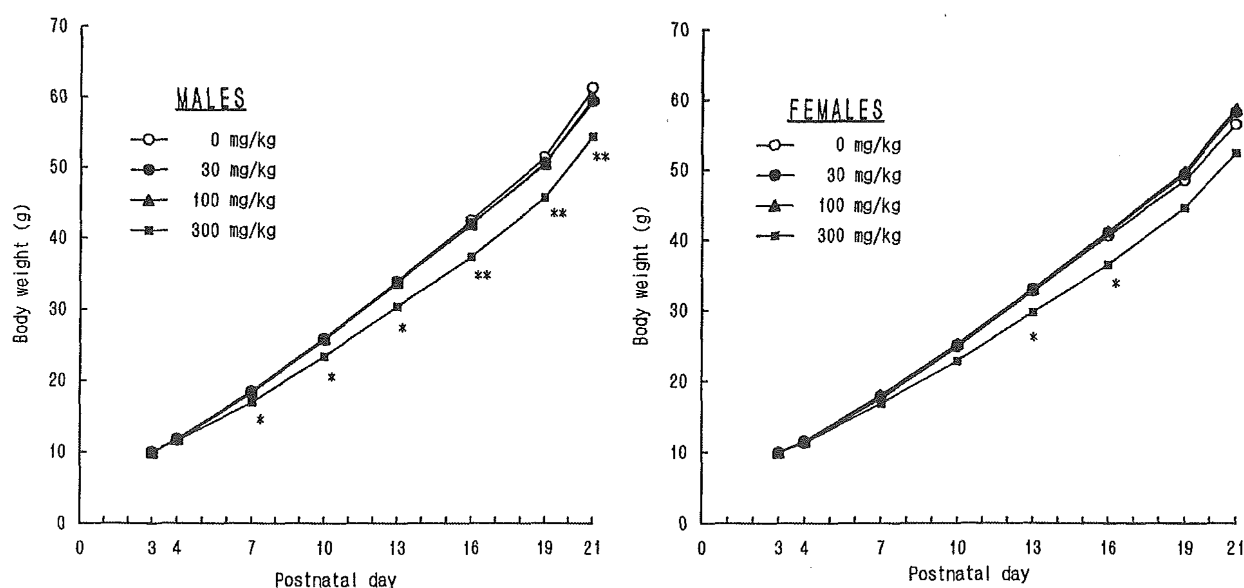

Fig. 1. Body weight change in 18-day study of 3-methylphenol in newborn rats (main study).
*: Significantly different from controls ($p < 0.05$), **: Significantly different from controls ($p < 0.01$).

(76.0 mg/dL, compared with 53.4 mg/dL at controls) and 500 mg/kg (72.2 mg/dL). Increase in relative liver weights in both sexes at 1,000 mg/kg (males: 3.86 g/100 g body weight, compared with 3.32 g/100 g body weight at controls, females: 3.70 g/100 g body weight, 3.34 g/100 g body weight) and 500 mg/kg (males: 3.60 g/100 g body weight, females: 3.68 g/100 g body weight), and in relative kidney weight in males at 1,000 mg/kg (0.47 g/100 g body weight, compared with 0.43 g/100 g body weight) was also observed. There were no dose-related changes evident on hematological and gross examination. Based on the results, the upper limit dose in the Test Guideline of 1,000 mg/kg was selected as the top dose for the main study, and 300 and 100 mg/kg were derived by division.

In the main study, deaths did not occur even at 1,000 mg/kg (Table 4). Salivation and tremors were observed throughout the dosing period at only 1,000 mg/kg in most males and females. At this dose, body weights were significantly lowered (finally 9% lower than controls for males and 11% for females) throughout the dosing period in males and from dosing day 14 in females, and food consumption was transiently lowered during the early dosing period in both sexes. At dosing week 4, increases in water consumption and urine volume were found in males and lowering of urinary pH in both sexes in the 1,000 mg/kg group. At 100 and 300 mg/kg, no changes in clinical signs, body weight, food consumption and urinalysis data were observed. Blood biochemical examination showed only slight increases in total cholesterol and BUN in males with a tendency for increase in total cholesterol in females receiving 1,000 mg/kg (Table 5). No dose-related changes in hematological findings were observed in any 3-methylphenol-treated group. There were significant increases in relative liver weights of

Table 2. Organ weights after 18-day repeat dosing of 3-methylphenol in newborn rats.

	Dose-finding study[a]			Main study			
Dose (mg/kg)	0	100	300	0	30	100	300
Males							
No. of animals	5	5	5	6	6	6	6
Body weight [b] (g)	62 ± 5	62 ± 6	59 ± 7	53.1 ± 3.3	52.7 ± 3.5	51.4 ± 3.5	46.7 ± 4.3*
Brain (g)	1.53 ± 0.05[c]	1.57 ± 0.06	1.52 ± 0.06	1.55 ± 0.04	1.58 ± 0.06	1.51 ± 0.06	1.47 ± 0.02*
	(2.47 ± 0.19[d])	(2.55 ± 0.18)	(2.61 ± 0.23)	(2.93 ± 0.18)	(3.00 ± 0.11)	(2.94 ± 0.13)	(3.16 ± 0.28)
Liver (g)	1.81 ± 0.13	1.91 ± 0.19	1.94 ± 0.24	1.74 ± 0.15	1.71 ± 0.13	1.75 ± 0.24	1.75 ± 0.20
	(2.90 ± 0.04)	(3.08 ± 0.19**)	(3.29 ± 0.05**)	(3.27 ± 0.12)	(3.24 ± 0.14)	(3.39 ± 0.25)	(3.74 ± 0.13**)
Kidney (g)	0.69 ± 0.06	0.72 ± 0.07	0.68 ± 0.05	0.64 ± 0.04	0.66 ± 0.06	0.62 ± 0.04	0.58 ± 0.02
	(1.11 ± 0.03)	(1.16 ± 0.03)	(1.16 ± 0.07)	(1.21 ± 0.05)	(1.26 ± 0.05)	(1.20 ± 0.06)	(1.25 ± 0.10)
Testis (mg)	310 ± 20	310 ± 30	320 ± 40	300 ± 28	293 ± 36	282 ± 14	270 ± 27
	(500 ± 20)	(500 ± 40)	(540 ± 20)	(566 ± 43)	(555 ± 52)	(549 ± 15)	(581 ± 51)
Females							
No. of animals	5	5	5	6	6	6	6
Body weight (g)	61 ± 4	59 ± 6	51 ± 6*	49.4 ± 3.8	50.5 ± 4.1	51.6 ± 3.3	45.5 ± 1.4
Brain (g)	1.50 ± 0.05	1.44 ± 0.03	1.43 ± 0.10	1.52 ± 0.05	1.48 ± 0.06	1.48 ± 0.05	1.42 ± 0.05*
	(2.46 ± 0.18)	(2.45 ± 0.24)	(2.81 ± 0.16*)	(3.09 ± 0.27)	(2.94 ± 0.27)	(2.88 ± 0.13)	(3.13 ± 0.10)
Liver (g)	1.77 ± 0.13	1.77 ± 0.12	1.67 ± 0.15	1.59 ± 0.18	1.59 ± 0.13	1.72 ± 0.08	1.61 ± 0.05
	(2.91 ± 0.08)	(3.01 ± 0.10)	(3.29 ± 0.15**)	(3.21 ± 0.13)	(3.16 ± 0.04)	(3.34 ± 0.11)	(3.54 ± 0.12**)
Kidney (g)	0.71 ± 0.05	0.70 ± 0.04	0.63 ± 0.06	0.63 ± 0.04	0.63 ± 0.04	0.65 ± 0.04	0.61 ± 0.04
	(1.19 ± 0.07)	(1.19 ± 0.10)	(1.24 ± 0.09)	(1.27 ± 0.03)	(1.25 ± 0.04)	(1.27 ± 0.09)	(1.34 ± 0.07)
Ovary (mg)	14.9 ± 2.8	14.4 ± 1.1	15.7 ± 2.4	15.6 ± 4.0	15.4 ± 3.0	13.8 ± 2.1	12.6 ± 2.3
	(24.4 ± 5.1)	(24.5 ± 1.5)	(30.9 ± 4.8)	(31.7 ± 8.9)	(30.5 ± 5.5)	(26.8 ± 4.7)	(27.8 ± 5.1)

Data are mean ± SD values.

[a]: In the 1,000 mg/kg group of the dose-finding study, since all animals died by dosing day 2, measurement of organ weights was not conducted, [b]: Body weight after overnight starvation following the last dosing, [c]: Absolute weight, [d]: Relative weight (g or mg/100 g body weight).

*: Significantly different from the control group ($p<0.05$), **: Significantly different from the control group ($p<0.01$).

both sexes at 1,000 mg/kg and of females at 300 mg/kg and in relative kidney weights of females at 1,000 mg/kg (Table 6). However, there was no change in absolute organ weights in any 3-methylphenol-treated group. On histopathological examination, no dose-related changes were observed in any of the 3-methylphenol-treated groups. At the end of the recovery period, no significant changes in any parameters were observed.

Table 3. Blood chemical findings after dosing period in 18-day study of 3-methylphenol in newborn rats (main study).

Dose (mg/kg)	0	30	100	300
Males				
No. of animals	6	6	6	6
GOT (IU/L)	127 ± 13	121 ± 7	121 ± 11	132 ± 22
GPT (IU/L)	24 ± 4	21 ± 4	21 ± 3	21 ± 3
γ-GTP (IU/L)	0.84 ± 0.24	0.90 ± 0.15	1.07 ± 0.11	1.19 ± 0.15**
Total bilirubin (mg/dL)	0.40 ± 0.03	0.41 ± 0.04	0.41 ± 0.03	0.47 ± 0.02**
Total cholesterol (mg/dL)	74 ± 11	78 ± 9	81 ± 7	85 ± 9
Triglyceride (mg/dL)	29 ± 10	25 ± 6	32 ± 3	28 ± 7
BUN (mg/dL)	13.5 ± 1.8	11.8 ± 2.1	13.0 ± 2.1	17.9 ± 3.6*
Females				
No. of animals	6	6	6	6
GOT (IU/L)	122 ± 15	119 ± 12	131 ± 9	116 ± 10
GPT (IU/L)	16 ± 2	19 ± 4	19 ± 4	17 ± 2
γ-GTP (IU/L)	0.93 ± 0.21	0.85 ± 0.10	0.98 ± 0.26	1.20 ± 0.14
Total bilirubin (mg/dL)	0.41 ± 0.04	0.40 ± 0.03	0.40 ± 0.02	0.45 ± 0.03
Total cholesterol (mg/dL)	77 ± 11	77 ± 10	75 ± 8	78 ± 12
Triglyceride (mg/dL)	24 ± 5	26 ± 2	25 ± 3	23 ± 3
BUN (mg/dL)	13.5 ± 2.3	13.5 ± 2.5	13.2 ± 2.3	14.2 ± 2.8

Data are mean ± SD values.
*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$).

Table 4. Clinical signs and mortality in repeated dose studies of 3-methylphenol in young rats.

	Dose-finding study (14-day)				Main study		
Dose (mg/kg)	125	250	500	1,000	100	300	1,000
Males							
No. of animals	5	5	5	5	7	7	14
No. of dead animals	–	–	–	–	–	–	–
No. of animals with clinical signs							
Salivation	–	–	–	3	–	–	11
Tremors	–	–	–	3	–	–	12
Prone/lateral position	–	–	–	1	–	–	1
Soiled perigenital fur	–	–	–	–	–	–	–
Females							
No. of animals	5	5	5	5	7	7	14
No. of dead animals	–	–	–	–	–	–	–
No. of animals with clinical signs							
Salivation	–	–	–	–	–	–	8
Tremors	–	–	–	4	–	–	13
Prone/lateral position	–	–	–	2	–	–	2
Soiled perigenital fur	–	–	–	–	–	–	2

–: No animals with clinical sign.

Table 5. Blood chemical findings after dosing period in repeated dose studies of 3-methylphenol in young rats (main study).

Dose (mg/kg)	0	100	300	1,000
Males				
No. of animals	7	7	7	7
GOT (IU/L)	68.6 ± 4.8	65.4 ± 5.4	62.7 ± 3.2	59.4 ± 5.4**
GPT (IU/L)	24.7 ± 2.9	25.4 ± 3.7	27.0 ± 2.9	28.0 ± 3.7
γ-GTP (IU/L)	0.17 ± 0.24	0.21 ± 0.13	0.60 ± 1.15	0.36 ± 0.23
Total bilirubin (mg/dL)	0.056 ± 0.005	0.049 ± 0.007	0.054 ± 0.010	0.050 ± 0.008
Total cholesterol (mg/dL)	52.7 ± 15.1	58.1 ± 11.8	58.3 ± 5.8	69.0 ± 9.4*
Triglyceride (mg/dL)	43.7 ± 19.8	54.7 ± 22.4	37.6 ± 3.1	50.0 ± 26.9
BUN (mg/dL)	13.89 ± 1.46	14.10 ± 0.85	14.56 ± 1.17	16.23 ± 2.14*
Females				
No. of animals	7	7	7	7
GOT (IU/L)	57.1 ± 4.3	65.9 ± 3.6	62.0 ± 5.7	59.1 ± 3.1
GPT (IU/L)	20.6 ± 2.2	21.4 ± 2.9	18.9 ± 3.1	20.1 ± 4.2
γ-GTP (IU/L)	0.83 ± 0.20	0.90 ± 0.16	1.00 ± 0.29	1.06 ± 0.10
Total bilirubin (mg/dL)	0.053 ± 0.011	0.056 ± 0.011	0.043 ± 0.008	0.054 ± 0.008
Total cholesterol (mg/dL)	63.4 ± 14.0	58.7 ± 10.6	61.4 ± 10.3	78.7 ± 13.7
Triglyceride (mg/dL)	15.4 ± 8.2	11.3 ± 4.8	9.9 ± 1.8	16.1 ± 5.2
BUN (mg/dL)	17.71 ± 1.96	16.63 ± 1.11	17.30 ± 2.14	18.03 ± 2.00

Data are mean ± SD values.
*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$).

Table 6. Organ weights after dosing period in repeated dose studies of 3-methylphenol in young rats (main study).

Dose (mg/kg)	0	100	300	1,000
Males				
No. of animals	7	7	7	7
Body weight[a] (g)	325.0 ± 23.5	345.6 ± 23.5	335.9 ± 16.7	298.3 ± 31.8
Brain (g)	2.04 ± 0.06[b]	2.11 ± 0.09	2.03 ± 0.08	2.05 ± 0.06
	(0.63 ± 0.05[c])	(0.61 ± 0.03)	(0.60 ± 0.02)	(0.69 ± 0.06*)
Liver (g)	10.55 ± 1.30	11.28 ± 1.08	11.29 ± 0.68	10.94 ± 2.01
	(3.24 ± 0.22)	(3.26 ± 0.19)	(3.36 ± 0.11)	(3.65 ± 0.34**)
Kidney (g)	2.65 ± 0.24	2.82 ± 0.24	2.78 ± 0.19	2.61 ± 0.23
	(0.82 ± 0.04)	(0.82 ± 0.03)	(0.83 ± 0.06)	(0.88 ± 0.05)
Testis (g)	2.96 ± 0.31	3.06 ± 0.21	2.95 ± 0.30	2.93 ± 0.22
	(0.91 ± 0.11)	(0.89 ± 0.08)	(0.88 ± 0.10)	(0.99 ± 0.14)
Females				
No. of animals	7	7	7	7
Body weight (g)	210.1 ± 15.4	207.6 ± 13.0	197.3 ± 19.3	186.4 ± 17.4*
Brain (g)	1.96 ± 0.06	1.90 ± 0.07	1.89 ± 0.07	1.88 ± 0.06
	(0.93 ± 0.06)	(0.92 ± 0.05)	(0.96 ± 0.08)	(1.01 ± 0.09)
Liver (g)	6.39 ± 0.68	6.59 ± 0.56	6.60 ± 0.67	6.51 ± 0.45
	(3.04 ± 0.17)	(3.17 ± 0.08)	(3.35 ± 0.13**)	(3.50 ± 0.20**)
Kidney (g)	1.66 ± 0.18	1.73 ± 0.11	1.65 ± 0.17	1.72 ± 0.14
	(0.79 ± 0.06)	(0.84 ± 0.05)	(0.84 ± 0.06)	(0.92 ± 0.03**)
Ovary (mg)	81.4 ± 13.7	82.0 ± 14.3	85.0 ± 14.0	78.9 ± 13.3
	(38.8 ± 6.3)	(39.5 ± 6.1)	(43.4 ± 8.3)	(42.4 ± 6.8)

Data are mean ± SD values.
[a]: Body weight after overnight starvation following last dosing, [b]: Absolute weight, [c]: Relative weight (g or mg/100 g body weight).
*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$).

Based on clinical signs of neurotoxicity with lowering of body weights, the unequivocally toxic level was concluded to be 1,000 mg/kg/day. Increase in relative liver weight without related changes at 300 mg/kg in the main study was not considered as an adverse effect. In the dose-finding study, effects on liver were noted at 500 mg/kg but no dose-related changes were evident at 250 mg/kg, which could not be taken into consideration of the estimation of the NOAEL because of the insufficient dosing period (14 days). Therefore, the NOAEL was concluded to be 300 mg/kg/day.

DISCUSSION

Concerning health of infants exposed to chemicals, our testing project has provided the following benefits. First, detailed examination of physical development and sexual maturation during the early postnatal period provides specific information on chemical toxicity towards newborn animals. Second, because the same experimental conditions, as much as possible, are set between newborn and young rat studies, this facilitates comparisons of toxicity. Furthermore, for toxicity levels, two additional analyses (estimation of unequivocally toxic levels in addition to NOAELs and careful incorporation of the dose-finding study) allow more precise / appropriate comparisons. So far, we have reported three comparative analyses of 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol (Koizumi *et al.*, 2001, 2002a, 2002b; Yamamoto *et al.*, 2001; Takano *et al.*, 2001; Nishimura *et al.*, 2002). As results, the toxicity profiles of these chemicals were similar in both ages, the susceptibility of newborn rats was 2 to 4 times higher than that of young rats, and no effects on physical development, sexual maturation and reflex ontogeny were observed.

In the present study, 3-methylphenol was selected as a fourth chemical. Clinical signs, indications of neurotoxicity to the central nervous system, were observed in both ages but not at the same dose level. Decrease in body weight gain also occurred in both ages but at a 3 times lower dose in newborn animals. In the newborn study, significant decrease in absolute brain weight was also evident at the highest dose, but no abnormalities on histopathology in the brain or in terms of functional development (reflex ontogeny) were observed. Brain weight changes were observed only in the groups showing 10% and more lowering of body weight and were not noted in the dose-finding study. Brain weight might be affected by decrease in body weight gain. As unequivocally toxic levels were clearly judged to be 300 mg/kg/day and 1,000 mg/kg/day for newborn and young studies, respectively, based on neurotoxic effects and decrease in body weight gain, newborn rats were considered to be approx. 3 times more susceptible to this chemical than young rats. NOAELs were concluded to be 30 mg/kg/day and 300 mg/kg/day for newborn and young rats, respectively, indicating a 10 times higher susceptibility in the newborn. However, tremors under contact stimulus were observed in only three males on single days and hypersensitivity on handling was noted only in one male on a single day in the 100 mg/kg newborns. Furthermore, no such toxic clinical signs were noted at 100 mg/kg in the dose-finding study under the same experimental conditions. It appears that the realistic no adverse effect dose for the newborn is slightly lower than 100 mg/kg/day rather than around 30 mg/kg/day. Based on this speculation and equal toxicity at the unequivocally toxic levels, the difference in the sensitivity to 3-methylphenol between newborn and young rats could be considered to be 3- to 4-fold.

As for the toxicity of 3-methylphenol, much information is available including unpublished data reported in reviews on this chemical or cresols (ATSDR, 1991; EHC, 1995; IRIS, 1997). In a 28-day feeding study (NTP, 1992), F344 rats were given diet containing 3-methylphenol at 0, 300, 1,000, 3,000, 10,000, 30,000 mg/kg diet. Depression of body weight gain, increase in relative liver and kidney weight and uterus atrophy were observed at 30,000 mg/kg diet (about 2,390 mg/kg/day). Increase in relative liver weight was also noted at 10,000 mg/kg diet (866 mg/kg/day). These results are consistent with our present results for young rats. However, clinical signs observed in our young rat study (daily administration by gavage) were not found at any doses in this NTP study, which might be due to the lower blood concentration with dietary application than in our gavage study. In a 90-day study (MBA, 1988), SD rats were administered 3-methylphenol by gavage at 50, 150, 450 mg/kg. In addition to depression of body weight gain at 150 mg/kg and more, a pronounced increase in the incidence of salivation, tremors and urination was observed at 450 mg/kg. In another 90-day study under the same test conditions with more detailed neurotoxic analysis, hypoactivity, rapid labored respiration and excessive salivation were observed sporadically in all treated groups, although few significant changes were found in performance on neurobehavioral test batteries, and no brain weight changes and no gross or histopathological changes in the brain or other nervous tissues (TRL,

1986). These clinical signs observed at lower doses than our young rat study might be due to the longer dosing period, but no information was provided on the incidence or dose-relationship. As for developmental toxicity, no effects on fetuses were observed in rats treated with 3-methylphenol by gavage at 450 mg/kg or less on days 6-15 of gestation (BRRC, 1988a). However, in a 2-generation reproductive toxicity study on rats by gavage (BRRC, 1989), some effects on pup body weights and survival (no details on the incidence and the degree) were evident with 450 mg/kg, which caused severe maternal toxicity including death and various clinical signs. There were occasional body weight changes in lower dose groups, but it is not clear whether these changes were treatment-related.

Some causes of differences in susceptibility of newborn and young rats to 3-methylphenol can be considered, such as specific physiological characteristics and immaturity of the brain-blood barrier and metabolism in the newborn. It is reported that 3-methylphenol is mainly eliminated as glucuronides in urine (Bray *et al.*, 1950). UDP-glucuronyltransferase activity in rat liver is known to be substrate-specific and generally low in neonates, and the activity against phenolic substances, p-nitrophenol and 1-naphthol, at birth has been shown to be comparable to adults but nearly 50% lower during the suckling period (exposure period in our newborn study) (Watkins and Klaassen, 1985; Rachmel and Hazelton, 1986). Therefore, the low capacity of glucuronidation might be one of the major causes for higher susceptibility of newborn rats to 3-methylphenol. In the case of humans, hepatic glucuronidation at birth is known to be relatively immature (Gow *et al.*, 2001), and it has been shown that *in vitro* bilirubin glucuronidation activity at birth is much lower than that of mature-phase values (Kawade and Onishi, 1981). These data suggest that human infants may be more susceptible to chemicals that are detoxified by this pathway.

The effects on the central nervous system, leading to death, are a major toxicological outcome characteristic of some phenolic compounds (Koizumi *et al.*, 2001, 2002b); however, the mechanism(s) responsible for eliciting neurotoxicity is unknown. As for hepatotoxicity, several studies on the mechanism and the structure activity relationship, using hepatocytes or liver slices, have been reported for three isomers of methylphenols and some para-alkylphenols (Bolton *et al.*, 1992; Thompson *et al.*, 1994, 1995, 1996; Kitagawa, 2001). In these studies, it has been shown that the quinone intermediates are most likely to be the causative agents for hepatotoxicity, possibly via mitochondrial toxicity, and the hepatotoxicity of alkylphenols depends on the position and the kind of alkyl groups with 4-methylphenol exerting the greatest degree of hepatotoxicity. In the case of 3-methylphenol, the neurotoxicity seems to be the most sensitive endpoint in both newborn and young animals, since only minor increases in relative liver weight have been observed without any histopathological changes.

In conclusion, 3-methylphenol showed the same toxicity profile—that is neurological symptoms and growth inhibition—in both newborn and young rats. However, the susceptibility of the newborn rats was 3 to 4 times higher than that of young rats, consistent with our previous results for three chemicals, 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol, which showed 2 to 4 times differences in susceptibility between newborn and young rats.

ACKNOWLEDGMENT

The authors gratefully acknowledge the financial support of the Office of Chemical Safety, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labor and Welfare, Japan.

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Arch Toxicol (2000) 74: 632–637
DOI 10.1007/s002040000163

DEVELOPMENTAL TOXICOLOGY

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Suppression of decidual cell response induced by tributyltin chloride in pseudopregnant rats: a cause of early embryonic loss

Received: 13 April 2000 / Accepted: 14 August 2000 / Published online: 9 November 2000
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Abstract In our previous studies, tributyltin chloride (TBTCl) at doses of 16.3 mg/kg and above caused implantation failure (preimplantation embryonic loss) and postimplantation embryonic loss in rats following administration on gestational day (GD) 0 through GD 3 and GD 4 through GD 7, respectively. This study was designed to assess the effects of TBTCl on uterine function as a cause of early embryonic loss in pseudopregnant rats. TBTCl was given orally to pseudopregnant rats at doses of 4.1, 8.1, 16.3 and 32.5 mg/kg on pseudopregnant day (PPD) 0 to PPD 3 or 8.1, 16.3, 32.5 and 65.1 mg/kg on PPD 4 to PPD 7. The decidual cell response was induced by bilateral scratch trauma on PPD 4. The uterine weight on PPD 9 served as an index of uterine decidualization. Uterine weight and serum progesterone levels on PPD 9 were significantly decreased after administration of TBTCl at doses of 16.3 mg/kg and above on PPD 0 to PPD 3 or PPD 4 to PPD 7. Administration of TBTCl at doses of 8.1 mg/kg and above on PPD 0 to 3 also significantly decreased serum progesterone levels on PPD 4. TBTCl had no effect on ovarian weight and number of corpora lutea. It can be concluded that TBTCl suppresses the uterine decidual cell response and decreases progesterone levels, and these effects are responsible for early embryonic loss due to TBTCl exposure.

Key words Tributyltin chloride · Decidual cell response · Pseudopregnancy · Rat

Introduction

Organotin compounds are a broad group of chemicals widely used both in agriculture and industry (Fait et al. 1994; Maguire 1991; Piver 1973; WHO 1980). Tributyltin (TBT) compounds have been used as antifouling agents, plastic stabilizers and wood preservative agents and in other applications (Fait et al. 1994; Piver 1973). The ubiquitous presence of TBT has been shown in the aquatic environment (Maguire 1991; Maguire et al. 1986). Food-chain bioamplification of TBT has been demonstrated in oysters (Waldock and Thain 1983), mud crabs (Evans and Laughlin 1984), marine mussels (Laughlin et al. 1986) and chinook salmon (Short and Thrower 1986). Daily intakes of TBT in Shiga prefecture in Japan, determined using the duplicate portion method, were 4.7 μg/day in 1991 and 2.2 μg/day in 1992 and, determined using the market basket method, 6.9 μg/day in 1991 and 6.7 μg/day in 1992 (Tsuda et al. 1995). The levels of organotin compounds in marine products were not sufficiently high to have any effect on human health (Tsuda et al. 1995; Ueno et al. 1999). However, more research on TBT residue levels in seafood is needed before a definitive conclusion on possible health risks can be drawn (Belfroid et al. 2000).

Several reports about the adverse effects of triorganotin compounds on mammal reproduction are available. Winek et al. (1978) have reported that triphenyltin hydroxide has an antifertility effect in rats orally dosed at 20 mg/kg on gestational day (GD) 1 through GD 7. Triphenyltin chloride (TPTCl) administered during early pregnancy causes implantation failure and has a greater antiimplantation effect when administered during earlier than later stages of blastogenesis (Ema et al. 1997). TPTCl exerts adverse effects on uterine decidualization in pseudopregnant rats together with a reduction in serum progesterone levels (Ema et al. 1999). We have previously reported that administration of TBT chloride (TBTCl) on GD 0 through GD 7 causes preimplantation embryonic loss (implantation failure) in female rats that had a positive mating (Harazono et al. 1996, 1998a). Furthermore, we have shown a differential susceptibility and manifestation of toxicity of TBTCl depending on the period of exposure and the dosage. TBTCl administered at doses of 16.3 mg/kg and above on GD 0

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through GD 3 and on GD 4 through GD 7 causes significant increases in the incidence of implantation failure (preimplantation embryonic loss) and postimplantation embryonic loss, respectively (Harazono et al. 1998b). In the present study, we determined the effects of TBTCl on uterine function for implantation and survival of embryos, as a cause of early embryonic loss, using a decidual cell response technique in pseudopregnant rats.

Materials and methods

Animals

Wistar rats (Jcl:Wistar, Clea Company, Tokyo, Japan) were used in this study. The animals were maintained in an air-conditioned room at 24±1 °C and at a relative humidity of 55±5%. A photoperiod of 12 h of light and 12 h of darkness was maintained, with the midpoint of the light phase set at 1300 hours. The rats were reared on a basal diet (F-1; Funabashi Farm Company, Funabashi, Japan) and had access to tap-water ad libitum. Vaginal smears of each rat were recorded daily, and only rats showing at least two consecutive 4-day cycles were used in this study. Virgin female rats of 12–15 weeks of age were mated overnight with vasectomized male rats of the same strain and inspected for the presence of a vaginal plug the following morning. The day when a vaginal plug was detected, coinciding with the last day of vaginal cornification, was considered to be pseudopregnant day (PPD) 0.

Decidual cell response

Traumatization of both uterine horns or a sham operation was performed via a midventral laparotomy under ether anesthesia between 1100 and 1300 hours on PPD 4. The decidual cell response was induced by scratching the antimesometrial surface of the endometrium with a bent needle. The bent needle was inserted into the uterine lumen at the bifurcation, passed up to the uterotubal junction and withdrawn (De Feo 1963).

Chemicals and dosing

The pseudopregnant rats were dosed once daily by gastric intubation with TBTCl (96% pure; Aldrich Chemical Company, Milwaukee, Wis.) at doses of 4.1, 8.1, 16.3 and 32.5 mg/kg (12.5, 25, 50 and 100 μmol/kg) on PPD 0 through PPD 3 or at doses of 8.1, 16.3, 32.5 and 65.1 mg/kg (25, 50, 100 and 200 μmol/kg) on PPD 4 through PPD 7. Doses of TBTCl were determined based on the results of our previous study, in which pre- and postimplantation embryonic loss were caused following maternal exposure to TBTCl at doses of 16.3 mg/kg and above on GD 0 through GD 3 and GD 4 through GD 7, respectively (Harazono et al. 1998b). TBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The volume of each dose was adjusted to 5 ml/kg of body weight based on daily body weight. The control, sham-operation and pair-fed rats received olive oil only. All TBTCl solutions were prepared fresh daily. The rats in the pair-fed group were given an amount of feed equal to that consumed by pseudopregnant rats in the highest dose group on PPD 0 to PPD 3 or PPD 4 to PPD 7.

Observations

The maternal body weight, food consumption and clinical signs of toxicity were recorded daily. The rats were killed on PPD 4 or PPD 9. The uterus and ovaries were weighed and the corpora lutea was counted. Uterine weight on PPD 9 served as an index of responsiveness of the uterus to deciduoma formation.

Analysis of steroid hormones

Blood samples were collected from the external jugular vein on the day of killing and serum was separated and stored at −80 °C for later assay of steroid hormones. Serum progesterone was measured by Shionogi Biomedical Laboratories (Shionogi and Company, Osaka, Japan) using kit materials for the radioimmunoassay (Diagnostic Products Corporation, Los Angeles, Calif.). The detection limit was 0.05 ng/ml. Serum estradiol was measured by SRL (Tokyo, Japan) using kit materials for the double antibody radioimmunoassay (Diagnostic Products Corporation). The detection limit was 1.4 pg/mg.

Data analysis

Data for all endpoints were analyzed by analysis of variance (ANOVA) and Dunnett's multiple comparison test. The 0.05 level of probability was used as the criterion for significance.

Results

Administration of TBTCl on PPD 0 to PPD 3

No deaths occurred in any group. Pseudopregnant rats treated with TBTCl at the higher doses showed sluggishness, reddish staining of the facial fur, diarrhea and/or piloerection, and the number of these rats increased with increasing dose.

Table 1 shows the body weight gain and food consumption in rats treated orally with TBTCl on PPD 0 to PPD 3 and killed on PPD 9. Body weight gain during

Table 1 Body weight gain and food consumption in female pseudopregnant rats treated orally with TBTCl on PPD 0 to PPD 3 and killed on PPD 9. Values are means ± SD

	Days	TBTCl dose (mg/kg)					Sham-operation	Pair-fed
		0 (control)	4.1	8.1	16.3	32.5		
No. of rats		10	10	10	10	10	10	10
Initial body weight (g)		220 ± 7	220 ± 6	221 ± 10	221 ± 9	221 ± 10	221 ± 11	221 ± 6
Body weight gain (g)	0–4	8 ± 4	−5 ± 12*,††	−10 ± 11**,††	−15 ± 14**,††	−20 ± 7**,††	9 ± 4	−32 ± 2**,††
	0–9	14 ± 5	11 ± 7	3 ± 10**,††	−1 ± 5**,††	−6 ± 8**,††	15 ± 3	−10 ± 3**,††
Food consumption (g)	0–4	47 ± 8	21 ± 12**,††	13 ± 11**,††	6 ± 5**,††	6 ± 2**,††	52 ± 4	5
	0–9	101 ± 11	85 ± 16*,††	70 ± 22**,††	66 ± 10**,††	59 ± 12**,††	117 ± 9	58

*$P < 0.05$, **$P < 0.01$ vs the control group; ††$P < 0.01$ vs the sham-operation group

PPD 0 to PPD 4 in rats treated with TBTCl at all doses and during PPD 0 to PPD 9 at doses of 8.1 mg/kg and above was significantly lower than that in the control and sham-operation groups. Food consumption during PPD 0 to PPD 4 and PPD 0 to PPD 9 in the TBTCl-treated groups was significantly lower than that in the control and sham-operation groups.

Uterine weight, serum progesterone levels, serum estradiol levels, ovarian weight and number of corpora lutea on PPD 9 in rats treated with TBTCl on PPD 0 to PPD 3 are presented in Fig. 1. Uterine weight was significantly decreased at doses of 16.3 mg/kg and above compared with the control value (Fig. 1A). Significant decreases in serum progesterone levels were observed at doses of 16.3 mg/kg and above compared with the control and sham-operation groups (Fig. 1B). Serum estradiol levels were increased in the TBTCl-treated groups, and the levels in the group treated at a dose of 32.5 mg/kg were significantly higher than those in the control and sham-operation groups (Fig. 1C). There were no differences in ovarian weight (Fig. 1D) or number of corpora lutea (Fig. 1E) among the groups. Uterine weight, serum progesterone and estradiol levels, ovarian weight and number of corpora lutea in the pair-fed group were not significantly different from those in the control group.

Fig. 1A–E Uterine weight, serum progesterone levels, serum estradiol levels, ovarian weight and number of corpora lutea on PPD 9 in pseudopregnant rats treated orally with TBTCl on PPD 0 to PPD 3. The values are means ± SEM from ten different rats (**A** uterine weight, **B** serum progesterone levels, **C** serum estradiol levels, **D** ovarian weight, **E** number of corpora lutea). $*P<0.05$, $**P<0.01$ vs control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs sham-operation

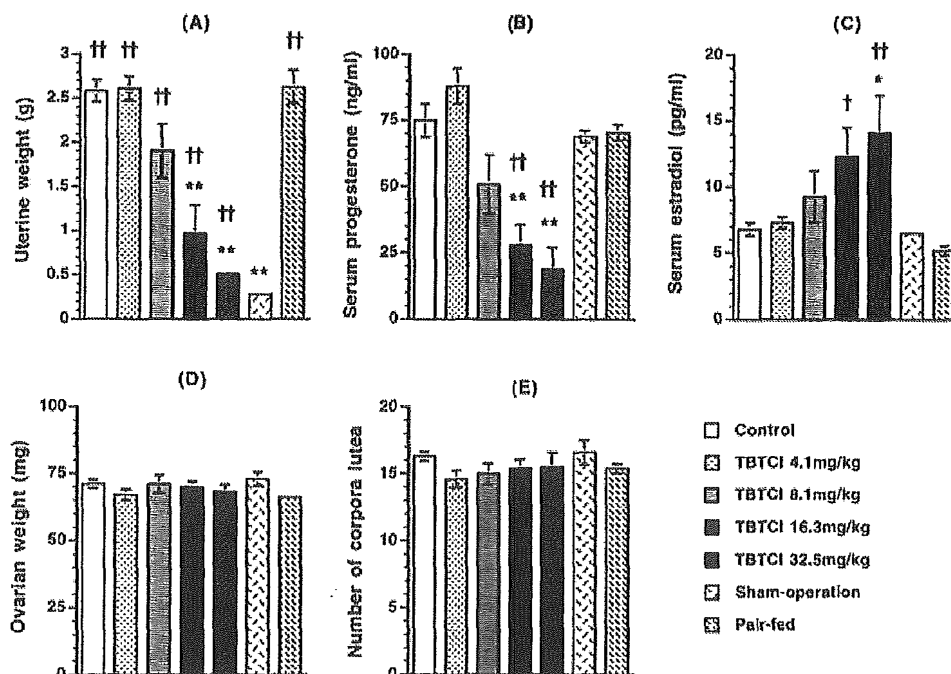
Figure 2 shows the serum progesterone and estradiol levels and ovarian weight on PPD 4 in rats treated with TBTCl on PPD 0 to PPD 3. The serum progesterone levels at doses of 8.1 mg/kg and above were significantly lower than the values in the control group (Fig. 2A). There were no differences in serum estradiol levels (Fig. 2B) and ovarian weight (Fig. 2C) between the TBTCl-treated and control groups. The serum progesterone and estradiol levels and ovarian weight in the pair-fed group were not significantly different from the values in the control group.

Administration of TBTCl on PPD 4 to PPD 7

No deaths occurred in any group. Pseudopregnant rats treated with TBTCl at the higher doses showed sluggishness, reddish staining of the facial fur, diarrhea and/or piloerection, and the number of these rats increased with increasing dose.

Table 2 shows the body weight gain and food consumption in rats treated with TBTCl on PPD 4 to PPD 7 and killed on PPD 9. The body weight gains during PPD 4 to PPD 8 in animals treated with TBTCl at doses of 16.3 mg/kg and above were significantly lower than those in the control and sham-operation groups. The food consumption during PPD 4 to PPD 8 in the TBTCl-treated groups was significantly lower than that in the control and sham-operation groups.

Uterine weight, serum progesterone levels, serum estradiol levels, ovarian weight and number of corpora lutea on PPD 9 in rats treated with TBTCl on PPD 4 to PPD 7 are presented in Fig. 3. Uterine weight was significantly decreased at doses of 16.3 mg/kg and above compared with that in the control group



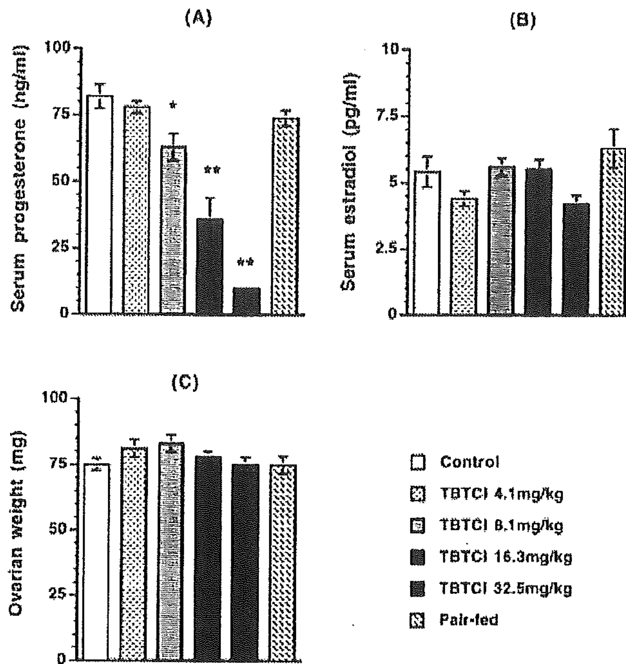


Fig. 2A–C Serum progesterone levels, serum estradiol levels and ovarian weight on PPD 4 in pseudopregnant rats treated orally with TBTCl on PPD 0 to PPD 3. The values are means ± SEM from 11 different rats in the control and 16.3 mg/kg groups and 10 different rats in the 4.1, 8.1, and 32.5 mg/kg groups (**A** serum progesterone levels, **B** serum estradiol levels, **C** ovarian weight). $^{*}P<0.05$, $^{**}P<0.01$ vs control

(Fig. 3A). A significant decrease in serum progesterone levels was observed at doses of 16.3 mg/kg and above compared with the control group and at 32.5 mg/kg and above compared with the sham-operation group (Fig. 3B). The serum estradiol levels at a dose of 32.5 mg/kg were significantly lower than in the control and sham-operation groups (Fig. 3C). There were no differences in ovarian weight (Fig. 3D) or number of corpora lutea (Fig. 3E) among the groups. Uterine weight, serum progesterone and estradiol levels, ovarian weight and number of corpora lutea in the pair-fed group were not significantly different from control values.

Discussion

We have previously reported that administration of TBTCl on GD 0 through GD 3 and GD 4 through GD 7 induces implantation failure and postimplantation embryonic loss, respectively (Harazono et al. 1998b). However, it cannot be deduced whether early embryonic loss results from direct effects of TBTCl on embryos and/or indirect effects perturbing homeostasis in the dams. The decidual cell response technique in pseudopregnant rats may help to distinguish between direct effects on the embryo and those that affect reproductive physiology (Cummings 1990).

Administration of TBTCl at doses of 16.3 mg/kg and above on PPD 0 to PPD 3 reduced the uterine weight on PPD 9 in a dose-dependent manner. This indicates that TBTCl could adversely affect the uterine decidualization. The decrease in serum progesterone levels induced by TBTCl was observed on PPD 4 and PPD 9. Development of uterine sensitivity and decidualization are strictly regulated by steroid hormones, progesterone and estradiol (Yochim and De Feo 1962, 1963). Thus, an inhibition of decidualization by TBTCl administered on PPD 0 to PPD 3 involves a blockade of the development of uterine sensitivity by decreased serum progesterone level.

TBTCl administration on PPD 0 to PPD 3 significantly increased serum estradiol levels on PPD 9 in a dose-dependent manner. However, the serum estradiol level on PPD 4 was not affected by TBTCl administration. It seems likely that the increase in the serum estradiol levels on PPD 9 was not a direct effect of TBTCl, but a secondary effect due to disturbance of endocrine homeostasis.

The present data are consistent with our previous findings showing that TBTCl at doses of 16.3 mg/kg and above produces a significant increase in the rate of implantation failure following administration during the preimplantation period (GD 0 through GD 3) (Harazono et al. 1998b). The dose levels of TBTCl inhibiting the decidual cell response are equivalent to those inducing complete inhibition of implantation when administered to pregnant rats. Consideration of these findings

Table 2 Body weight gain and food consumption in female pseudopregnant rats treated orally with TBTCl on PPD 4 to PPD 7 and killed on PPD 9. Values are means ± SD

	Days	TBTCl dose (mg/kg)					Sham-operation	Pair-fed
		0 (control)	8.1	16.3	32.5	65.1		
No. of rats		10	10	10	10	10	10	10
Initial body weight (g)		220 ± 8	220 ± 8	220 ± 4	219 ± 4	220 ± 9	220 ± 5	220 ± 3
Body weight gain (g)	4–8	0 ± 4	0 ± 7	$-11 \pm 7^{**,\dagger\dagger}$	$-13 \pm 7^{**,\dagger\dagger}$	$-17 \pm 8^{**,\dagger\dagger}$	2 ± 2	$-17 \pm 2^{**,\dagger\dagger}$
	0–9	14 ± 4	14 ± 6	5 ± 9	$-1 \pm 10^{**,\dagger\dagger}$	$-4 \pm 11^{**,\dagger\dagger}$	15 ± 4	$-6 \pm 4^{**,\dagger\dagger}$
Food consumption (g)	4–8	35 ± 5	$24 \pm 8^{**,\dagger\dagger}$	$13 \pm 9^{**,\dagger\dagger}$	$12 \pm 6^{**,}$	$14 \pm 5^{**,\dagger\dagger}$	41 ± 11	14
	0–9	113 ± 9	102 ± 13	$84 \pm 14^{**,\dagger\dagger}$	$82 \pm 10^{**,\dagger\dagger}$	$84 \pm 13^{**,\dagger\dagger}$	125 ± 8	$84 \pm 4^{**,\dagger\dagger}$

$^{**}P<0.01$ vs the control group; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs the sham-operation group

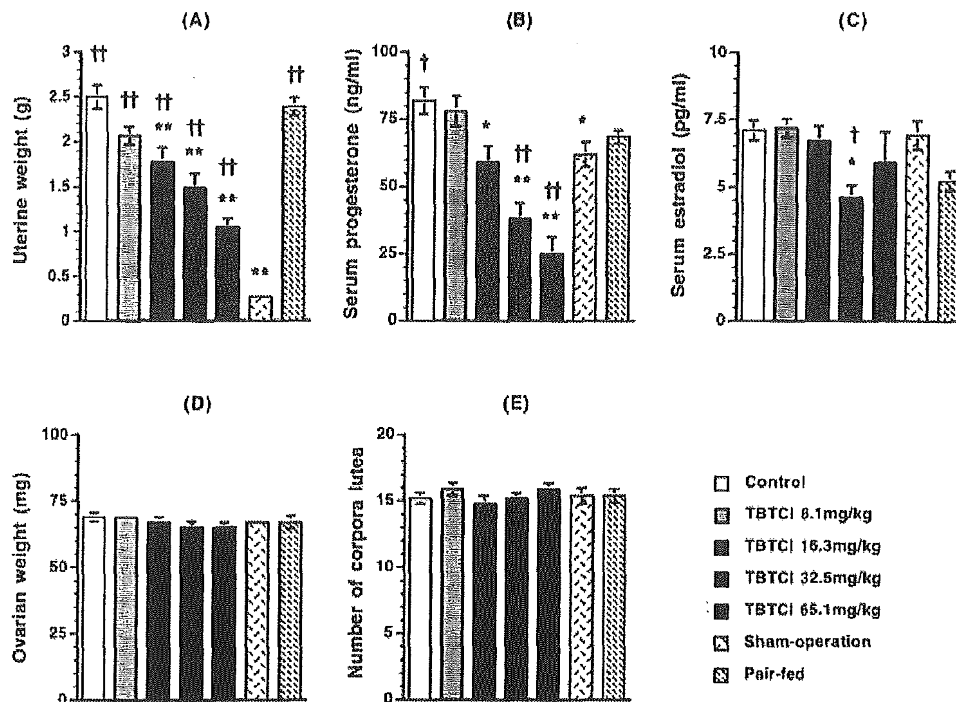


Fig. 3A–E Uterine weight, serum progesterone levels, serum estradiol levels, ovarian weight and number of corpora lutea on PPD 9 in pseudopregnant rats treated orally with TBTCl on PPD 4 to PPD 7. The values are the means ± SEM from ten different rats (**A** uterine weight, **B** serum progesterone levels, **C** serum estradiol levels, **D** ovarian weight, **E** number of corpora lutea). $*P<0.05$, $**P<0.01$ vs control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs sham-operation

together indicates that the suppression of the responsiveness of the uterus to deciduoma formation is responsible for implantation failure after administration of TBTCl.

Administration of TBTCl at doses of 16.3 mg/kg and above on PPD 4 to PPD 7 reduced the uterine weight on PPD 9, and this was correlated with a reduction in serum progesterone levels, suggesting that TBTCl could suppress decidua formation. Hashimoto et al. (1968) have shown that ovarian progesterone secretion is higher in deciduoma-bearing rats than in pseudopregnant rats without decidual tissue. The possibility may remain that the decreases in serum progesterone levels were caused by suppression of deciduoma growth. Progesterone levels on PPD 9 in the TBTCl-treated groups were also significantly decreased compared with those in the sham-operation group, indicating no induction of decidual cell response. These findings suggest that the decrease in progesterone levels in the TBTCl-treated groups may have been due to an effect of TBTCl itself but not to a reduction in the endocrine activity (luteotrophic influence) of the decidual tissue by suppression of decidualization. The reduction in serum progesterone levels may be the primary factor mediating suppression of the decidual cell response by TBTCl since adequate levels of progesterone are required for normal uterine function and decidual growth (Yochim and De Feo 1962).

In our previous study, administration of TBTCl at doses of 16.3 mg/kg and above on GD 4 through GD 7 produced postimplantation embryonic loss and at 65.2 mg/kg implantation failure, as indicated by the total absence of an implantation site (Harazono et al. 1998b). It appears that inhibition of the decidual response may be related to an increase in the incidence of postimplantation embryonic loss and implantation failure.

In order to determine the effect of significantly low food consumption and body weight on uterine weight and serum progesterone levels, the pseudopregnant rats in the pair-fed group were given an amount of feed equal to the feed consumption of rats in the highest dose group of each dosing period. None of the parameters measured in the pair-fed group was significantly different from the control value excluding maternal weight gain and food intake. This indicates that suppression of the decidual cell response observed in the TBTCl-treated groups was due to the effects of TBTCl and not due to malnutrition or reduced body weight.

Administration of TBTCl at doses of 16.3 mg/kg and above decreased serum progesterone levels, regardless of the days of administration of TBTCl. Further work is necessary to determine the effect of TBTCl on ovarian progesterone production.

In summary, the present study demonstrated that TBTCl induced suppression of the decidual cell response in pseudopregnant rats, concurrent with a decrease in serum progesterone levels, suggesting that TBTCl administered during early pregnancy adversely affects uterine decidualization, mediated by a decrease in serum progesterone levels. This inhibition of the decidual cell response may be responsible for the blockade of im-

plantation and induction of embryonic resorption in pregnant rats given TBTCl during early pregnancy.

Acknowledgements This study was supported in part by a grant from the Environment Agency in Japan.

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Periostin is an extracellular matrix protein required for eruption of incisors in mice

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Received 27 January 2006
Available online 14 February 2006

Abstract

A characteristic tooth of rodents, the incisor continuously grows throughout life by the constant formation of dentin and enamel. Continuous eruption of the incisor is accompanied with formation of shear zone, in which the periodontal ligament is remodeled. Although the shear zone plays a role in the remodeling, its molecular biological aspect is barely understood. Here, we show that periostin is essential for formation of the shear zone. *Periostin*$^{-/-}$ mice showed an eruption disturbance of incisors. Histological observation revealed that deletion of *periostin* led to disappearance of the shear zone. Electron microscopy revealed that the disappearance of the shear zone resulted from a failure in digestion of collagen fibers in the *periostin*$^{-/-}$ mice. Furthermore, immunohistochemical analysis using anti-periostin antibodies demonstrated the restricted localization of periostin protein in the shear zone. Periostin is an extracellular matrix protein, and immunoelectron microscopy showed a close association of periostin with collagen fibrils in vivo. These results suggest that periostin functions in the remodeling of collagen matrix in the shear zone.
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Keywords: Periostin; Incisor; Collagen; Eruption; Periodontal ligament; Shear zone; Tooth; Fasciclin

The periodontal ligament is a dense connective tissue containing fibroblastic cells and vascular and neural elements [1,2]. Thick collagen bundles, i.e., principal periodontal fibers, run between the cementum and alveolar bone, and some of them penetrate into these tissues as Sharpey's fibers. The periodontal ligament serves as a cushion as well as an anchor between the teeth and the alveolar bone during mastication. Tooth eruption and orthodontic tooth movement induce the active remodeling of the periodontal ligament, following to realignment of teeth. Several MMPs are reported to be involved in the remodeling of the periodontal ligament [3–7].

Rodent's incisors, having a sharp chisel-like shape, differ from other teeth, molars, in certain biological properties: incisors erupt continuously throughout life. The labial portion of these teeth is covered with enamel, and the remaining surface with cementum. Previously, it was reported that the periodontal ligament of the rodent incisor comprises two compartments: a highly vascularized part and an unvascularized part, and these are also called the alveolus-related part and the tooth-related part, respectively [8]. The former, which localizes closer to the alveolar bone, is regarded as the non-moving zone, whereas the latter is the zone that moves during continuous incisor eruption. The boundary between these alveolus- and tooth-related parts is referred to as the shear zone [8]. Synthesis and turnover of total proteins occur throughout the periodontal ligament, whereas remodeling of collagens predominantly occurs in the shear zone [9]. Although the shear zone plays an important role in the remodeling of the periodontal ligament, its molecular biological aspect is barely understood.

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doi:10.1016/j.bbrc.2006.02.016

In the previous studies, we succeeded in identifying an extracellular matrix protein that we termed periostin because of its specific expression patterns in the periosteum and periodontal ligament in adult mice [10–12]. In addition, observations on the fetal mouse demonstrated the expression of periostin in the fascias of muscles, perichondrium, articular surfaces of the epiphyseal cartilage, and joint ligaments [13,14]. Thus, periostin expression is temporally regulated and spatially restricted mostly to the cells of connective tissues, suggesting its potential role in the formation and maintenance of the tendinous connective tissue structures.

In this study, we found that *periostin*$^{-/-}$ mice showed an eruption disturbance of incisors and intended to obtain a mechanistic insight into how the shear zone was maintained in the continuous eruption of incisors.

Materials and methods

Animals. The *periostin*$^{-/-}$ mouse was generated in our laboratory (Kii et al. [15] and Shimazaki et al., manuscript in preparation). Simply, we performed targeted disruption of the periostin gene in mouse embryonic stem (ES) cells using homologous recombination, and the inserted Neo gene was finally deleted in deficient mice by crossing with CAG-Cre mice to excise the neo cassette, and no periostin expression was observed. The *periostin*$^{-/-}$ mice were viable, and the newborns appeared normal. All animals were allowed free access to standard mouse chow and water during the whole experimental period.

Histological analysis. We used four 6-week-old male mice and six 12-week-old male mice homozygous for the disrupted *periostin* gene, and the same number of their wild-type littermates. The mice were perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The mandibles were removed and immersed in the same fixative for an additional 12 h and then decalcified with EDTA solution for 3 weeks. Paraffin sections were prepared and incubated with an antiserum against mouse periostin. Final visualization of immuno-reaction sites was accomplished with 3-3′ diaminobenzidine. Rabbit anti-periostin antibodies were previously described [12].

Immunoelectron microscopy. Cryostat sections obtained from fixed and decalcified specimens were incubated with rabbit polyclonal antisera against mouse periostin and subsequently with HRP-conjugated secondary antibody. The immuno-reactions were visualized with 3-3′ diaminobenzidine. For immunoelectron microscopy, the specimens were processed as described previously [16].

Eruption experiment. To determine if *periostin* would be physiologically involved in the eruption of the incisors, we cut off one of the two lower incisors in the wild-type and the *periostin*$^{-/-}$ mice at the gingival margin. The erupted incisors were then observed 4 days post-operation.

Results

Eruption disturbance of incisors in the periostin$^{-/-}$ *mice*

The *periostin*$^{-/-}$ mice were viable, and the newborns appeared normal. However, as early as 6 weeks after birth, the incisors from the *periostin*$^{-/-}$ mice were shorter than those from the wild-type littermates (data not shown). In 12-week-old wild-type mice, their incisors were nearly adjoined, with only a narrow space between them (Fig. 1A). In contrast, in the 12-week-old *periostin*$^{-/-}$ mice, lower incisors were shorter than those in the wild-type littermates, and the space between the incisors was wider (Fig. 1B), furthermore, they displayed a chalky white color,

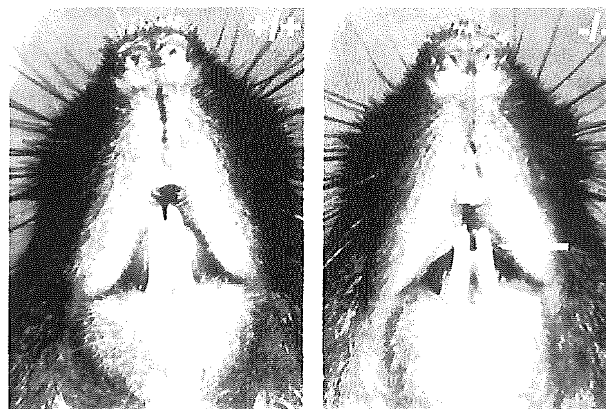

Fig. 1. Abnormal appearance of incisors in the *periostin*$^{-/-}$ mice. Photographs of incisors in 12-week-old wild-type (A) and *periostin*$^{-/-}$ (B) mice. The arrow in (B) indicates short and chalky white incisors in this *periostin*$^{-/-}$ mouse.

indicating disorganization of the enamel layers (Fig. 1B, arrow). Upper incisors of the 12-week-old *periostin*$^{-/-}$ mice also showed similar abnormal phenotypes.

To further investigate whether *periostin* is physiologically involved in eruption of incisors, we cut off one of two lower incisors in the 12-week-old wild-type and the *periostin*$^{-/-}$ mice at the gingival margin, and observed the eruption of incisors 4 days post-operation. In the *periostin*$^{-/-}$ mice, the eruption of the incisor was severely disrupted (Fig. 2B), while in the wild-type mice it normally occurred

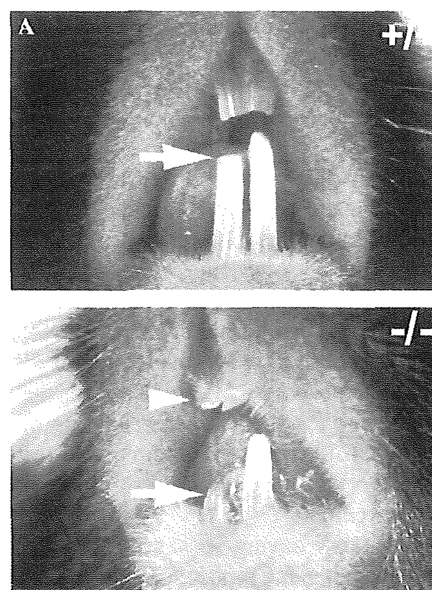

Fig. 2. Eruption disturbance of incisors in the *periostin*$^{-/-}$ mice. Photographs showing incisors that erupted 4-days post-amputation in 12-week-old wild-type (A) and *periostin*$^{-/-}$ (B) mice. In the wild-type mouse, the amputated incisor (left) erupted to the same degree as the intact incisor (right): whereas in the *periostin*$^{-/-}$ animal, the amputated incisor (left) barely erupted. Arrows indicate top of the amputated incisors. Abnormal teeth in the *periostin*$^{-/-}$ mouse are also noted (arrowhead).

(Fig. 2A). These results indicate that periostin acts on the eruption of the incisors.

Disorganization of enamel and dentin layers in the periostin$^{-/-}$ incisors

To investigate the tissue formation in the *periostin*$^{-/-}$ incisors, we performed the histological analyses at the apical region of the incisors. The incisors of rodents are permanently built from the apical bud, and are pushed out anteriorly. In the histological analysis of the apical region in the 6-week-old *periostin*$^{-/-}$ mice, the obvious focal disorganization of ameloblastic layers and the thick dentin and enamel layers, which were waved gently, were observed (Fig. 3B, arrow), compared with those tissues in the wild-type mice (Fig. 3A), although in the *periostin*$^{-/-}$ mice the apical bud generating dental pulp, dentin, and enamel was observed. In the 12-week-old *periostin*$^{-/-}$ mice, the incisal enamel and dentin layers were compressed and undulated (Fig. 3D), though in the wild-type mice they were thin and smooth (Fig. 3C). These findings imply that the incisors in the *periostin*$^{-/-}$ mice became more compressed and undulated from 6- to 12-week-old mice.

In rodents, tooth development, enamel covering of tooth is built in four distinct steps. The first step is the secretion of extracellular matrices and the initiation of enamel crystal growth by polarized ameloblasts (the secretory stage). The second step is the matrix degradation and the differentiation of ameloblasts (the transition stage). The third step is the removal of residual protein components and the crystal growth (the maturation stage). The fourth step is the complete of covering with enamel (the mature stage). These enamel formation processes are found on each incisor of adult mice from the apical region to the front orderly, because of the continuous enamel formation due to the growth and eruption of incisors. High magnification of the ameloblastic layer of the 12-week-old wild-type mice showed a dense cell layer consisting of polarized ameloblasts (nuclei located proximally) in the secretory stage (Fig. 3E), and ameloblasts in the maturation stage (Fig. 3G). In the 12-week-old *periostin*$^{-/-}$ mice, this cell layer was loosened, and partial non-polarized ameloblasts were observed in the secretory stage (Fig. 3F). Moreover, the gaps between the polarized ameloblasts appeared in the maturation stage (Fig. 3H). These histological observations demonstrated that in the *periostin*$^{-/-}$ mice, the morphology and alignment of the ameloblasts were severely disrupted, indicating the impaired enamel layer formation in the absence of *periostin* gene.

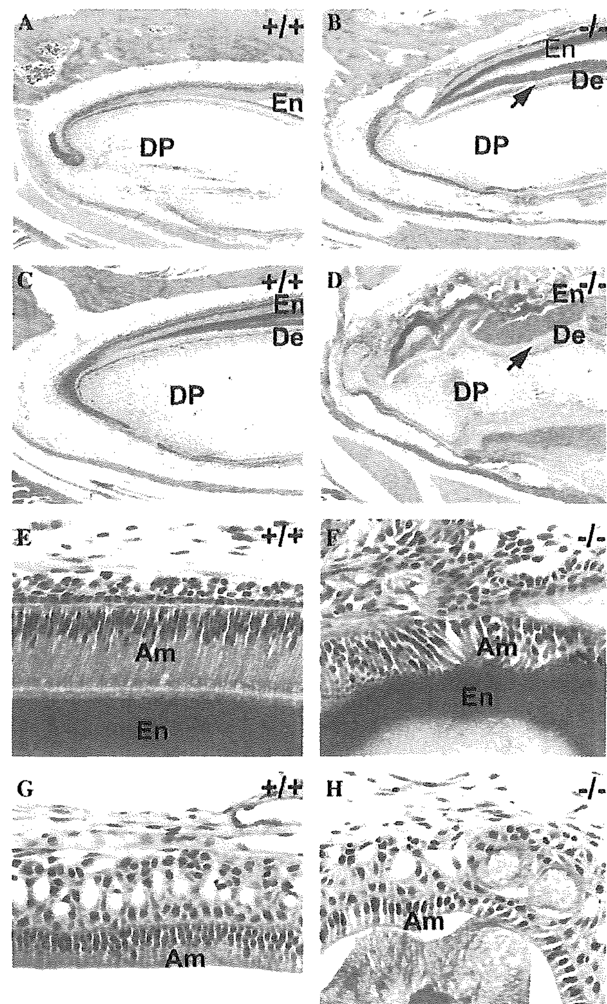

Fig. 3. Histological analysis of the apical region of incisors in the *periostin*$^{-/-}$ mice. Representative histological sections (hematoxylin/eosin stain) of the apical region (root) of incisors in the mandibles from 6-week- (A,B) and 12-week- (C,D) old wild-type (+/+) and *periostin*$^{-/-}$ (−/−) mice show that incisal enamel and dentin are compressed and undulated in the *periostin*$^{-/-}$ mice (arrows). Histological sections (hematoxylin/eosin staining) at high magnifications of the secretory stage (E,F) and the maturation stage (G,H) of enamel in 12-week-old wild-type (+/+) and *periostin*$^{-/-}$ (−/−) mice show disorganized enamel layers in the *periostin*$^{-/-}$ mice. DP, dental pulp; En, enamel; De, dentin; Am, ameloblastic layer.

The shear zone in the periostin$^{-/-}$ mice

Furthermore, we examined the immunohistological localization of periostin protein at the apical region of the incisors in the 12-week-old wild-type mice. A positive immunoreaction for periostin was observed only in the periodontal ligament of incisors (Fig. 4). At a high magnification, the strong immuno-reactivity for periostin was observed in the middle region of the periodontal ligament corresponding to the shear zone that is the boundary between tooth- and alveolus-related parts (Fig. 4C, arrows), whereas a faint staining was observed in the periodontal ligament close to the cementum and the alveolar bone surface (Fig. 4C). Thus, this strong immuno-reactivity for periostin in the shear zone indicates a role for periostin protein in the shear zone for eruption of the incisors.

To address the requirement for periostin protein in the shear zone, we histologically analyzed sections of the periodontal ligament around the incisors in the *periostin*$^{-/-}$ mice. The shear zone was clearly distinguishable