EFFECTS OF ALL-TRANS-RETINOIC ACID ON LIMB DEVELOPMENT IN THE GENETIC POLYDACTYLY MOUSE

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ABSTRACT — *Pdn/+* female mice, mated with *Pdn/+* males, were treated with 40 mg/kg body weight of all-*trans*-retinoic acid (RA) intraperitoneally on day 10 or 11 of gestation, and effects on the limb development were investigated.

RA treatment induced the shortening of stylopodium and zygopodium. In the present experiment, we focused on the differences between genotypes in the shortening of stylopodium and zygopodium induced by RA. The effects of RA were milder in *Pdn/Pdn* than +/- (*Pdn/+* and/or *+/+*) fetuses. The differences between genotypes in the effects of RA were more significant in the group treated on day 11 than on day 10 of gestation.

Cartilage of stylopodium and zygopodium was longer in day-13 *Pdn/Pdn* embryos exposed to RA on day 11 of gestation than those in similarly treated +/- embryos. Many apoptotic cells were observed in the mesenchyme of the forelimb plates at 12 hr after injection of RA on day 11 of gestation.

These results suggest that the *Pdn* gene might influence the apoptosis induced by RA in the mesenchymal cells of the limb, causing milder effects in the shortening of stylopodium and zygopodium in *Pdn/Pdn* fetuses.

KEY WORDS : *Pdn* mice, Retinoic acid, Apoptosis, Limb anomaly.

INTRODUCTION

13-*cis*-retinoic acid has been shown to be a potent teratogen in humans (Rosa, 1983 ; Lammer *et al.*, 1985). All-*trans*-retinoic acid (RA) was demonstrated to induce neural tube defects in mice (Kochhar, 1967 ; Yasuda *et al.*, 1986). Yasuda *et al.* (1989) suggested that RA induced not only cytotoxicity but also dedifferentiation in the neuroepithelial cells leading to more cell death, which activated the phagocytosis, and

these lesions in the neuroepithelium might be a cause of exencephaly. It is also well known that maternal RA exposure induces mesomelic abnormalities of the limbs in mice (Kochhar, 1977).

Homozygous Polydactyly Nagoya (*Pdn/Pdn*) mice exhibit preaxial polydactyly of duplicated or triplicated metacarpal/metatarsal type in both the fore- and hindlimbs. In these mice, the radius and ulna are normal, but the tibia and fibula are slightly shorter than normal. Heterozygotes (*Pdn/+*) have one extra digit of distal phalangeal type preaxially in the hindlimbs and deformity of the distal phalanx of the first digit in the forelimbs (Naruse and Kameyama, 1982).

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It has been reported that RA administration to pregnant mice alters the phenotypes of the progeny in various mutant strains (Kapron-Brás and Trasler, 1984; Cusic and Dagg, 1985; Katter, 1985; Scott *et al.*, 1994). In the present study, alterations of the phenotypes of the genetic polydactyly mouse (*Pdn*) by RA were investigated.

MATERIALS AND METHODS

Animals were maintained at a temperature of $22 \pm 1^\circ\text{C}$ and humidity of $55 \pm 10\%$ with a 12-hr light (6:00–18:00)/dark cycle. Female *Pdn*/+ mice, older than 2 months of age, were caged in pairs overnight with *Pdn*/+ males. Pregnancy was dated as day 0 if a vaginal plug was observed in the morning. Pregnant female was housed individually in a plastic cage (190×260×125 mm) with wood chips for bedding, and fed NMF pellet diet for reproduction (Oriental Yeast) and tap water *ad libitum*. All-*trans*-retinoic acid (RA, Sigma) at a concentration of 4 mg/ml was suspended in olive oil (Katayama Chemical) and pregnant females were treated with 40 mg/kg (body weight) of RA intraperitoneally on day 10 or 11 of gestation. The control dams were treated with 10 ml/kg (body weight) of olive oil alone on day 11 of gestation. The dams were sacrificed by cervical dislocation and the uterine horns were removed on day 18 of gestation. The numbers of implantations, live fetuses and resorptions, fetal body weight and sex ratio were recorded. External malformations were observed grossly. *Pdn/Pdn* fetuses have no olfactory bulb (Naruse *et al.*, 1990). To determine the genotypes, cranial skin of the live fetus was detached to observe the olfactory bulbs. Fetuses which had no olfactory bulb were regarded as *Pdn/Pdn*, and those which had olfactory bulbs were regarded as +/- (*Pdn*/+ and/or +/+). Since it was impossible to distinguish *Pdn*/+ from +/+ after RA treatment, *Pdn*/+ and +/+ were grouped together as +/- . The fetuses were fixed with 95% alcohol after peeling their skin, and bones and cartilages were then stained with alcian blue/alizarin red S according to Inouye (1976). Bone and cartilage specimens were observed, and the lengths (maximum diameter) of humerus, radius, ulna, femur, tibia and

fibula were measured under a dissecting microscope.

The forelimbs of *Pdn/Pdn* and +/- embryos treated with RA on day 11 were fixed in 10% neutral buffered formalin on day 13 of gestation. Cartilage was stained with 1% methylene blue and cleared by methyl benzoate and benzene.

The forelimbs at 12 hr after treatment with olive oil or RA on day 11 were fixed in 5% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 10 μm . Sections were stained with hematoxylin and eosin or with an antibody against single-stranded DNA using the avidin-biotin-horseradish peroxidase complex (ABC Elite Kit, Vector) method. The antibody against single-stranded DNA (Yamada *et al.*, 1985) was specific for the detection of nuclei of drug-induced apoptotic cells and programmed dying cells (Naruse *et al.*, 1994).

Statistical analyses concerning the number of digital bones, and malformations and lengths of stylopodium and zygapodium were conducted using the limb as sample unit, and the other parameters using the fetus as sample unit. The incidences of postimplantation loss and each type of malformations were analyzed using Fisher's exact test. Fetal body weight and the lengths of stylopodium and zygapodium were analyzed using Student's *t*-test or Aspin-Welch test, and the number of digital bones was analyzed using Wilcoxon's rank-sum test. Two-way ANOVA was employed to analyze the interaction between genotype and RA treatment in the lengths of stylopodium and zygapodium. Since unilateral predominant abnormalities were not observed in the present experiment, the data on right and left limbs were grouped together.

RESULTS

Effects of RA on reproduction (Table 1): The incidences of postimplantation loss, cleft palate and tail abnormalities were increased in both RA-treated groups. Body weight of female fetuses was decreased by RA treatment on day 11 of gestation.

Effects of RA on the number of digital bones (Table 2): In +/- fetuses, RA treatment on day 11 induced the decrease of the numbers in metacarpals, proximal and middle phalanges in

Table 1. Reproductive data in *Pdn* mice treated with all-*trans*-retinoic acid.

	Vehicle control olive oil 10 ml/kg on day 11	Retinoic acid 40 mg/kg on day 10	Retinoic acid 40 mg/kg on day 11
No. of dams	10	10	10
No. of implantations	133	127	124
No. of live fetuses	89	66	55
No. of resorptions	44	61	69
Postimplantation loss (%)	33.1	48.0*	55.6***
Male/Female	45/44	35/31	23/32
Fetal body weight (g $\pm$ S.D.)			
Male	1.32 $\pm$ 0.141	1.29 $\pm$ 0.199	1.27 $\pm$ 0.136
Female	1.24 $\pm$ 0.140	1.27 $\pm$ 0.225	1.12 $\pm$ 0.191**
No. of +/- (<i>Pdn</i> /+ and/or +/+) fetuses	67	49	46
No. of <i>Pdn/Pdn</i> fetuses	22	17	9
External malformations except for the limbs			
Exencephaly	5 (5.6%)	3 (4.5%)	3 (5.5%)
Open eyelids	7 (7.9%)	4 (6.1%)	1 (1.8%)
Cleft palate	4 (4.5%)	33 (50.5%)*	46 (83.5%)*
Tail abnormalities	0	23 (34.8%)*	8 (14.5%)*

**** : Significantly different from vehicle control by Fisher's exact test ($P < 0.05$, 0.001).

** : Significantly different from vehicle control by Student's *t*-test or Aspin-Welch test ($P < 0.01$).

the forelimbs, and in the metatarsals and all phalanges in the hindlimbs. RA treatment on day 10 also induced the decrease of the numbers in metacarpals and middle phalanges in the forelimbs, and in the middle and distal phalanges in the hindlimbs. The number of distal phalanges in the forelimbs were increased in both RA-treated groups.

On the other hand, no alterations were observed in *Pdn/Pdn* fetuses treated with RA on either day.

Effects of RA on the development of stylopodium and zygapodium (Table 3): In +/- fetuses, increases in the incidence of deformation (bending, shortening and thickening), absence and synostosis of stylopodium and zygapodium were induced by RA treatment on day 11 of gestation. The same types of malformations except for femur-tibia synostosis were induced by RA treatment on day 10 of gestation. The incidences of malformations induced by RA treatment were higher in the group treated on day 11 than on day

10 of gestation.

In *Pdn/Pdn* fetuses, the incidences of deformed humerus, radius and ulna were increased in both RA-treated groups, and increased rates of deformed femur and fibula were observed following RA treatment on day 11 of gestation. The incidences of deformed humerus, radius and ulna induced by RA treatment were higher in the group treated on day 11 than on day 10 of gestation. The absence of fibula and femur-tibia synostosis were not increased in either RA-treated group.

The incidences of malformations of stylopodium and zygapodium had a tendency to be lower in *Pdn/Pdn* than +/- fetuses in both RA-treated groups.

Effects of RA on the lengths of stylopodium and zygapodium (Table 4): In +/- fetuses, all stylopodium and zygapodium were shortened in both RA-treated groups, and especially severe shortening or absence were induced by the treatment on day 11 of gestation.

Table 2. Number of digital bones in *Pdn* mice treated with all-*trans*-retinoic acid.

	Vehicle control olive oil 10 ml/kg on day 11	Retinoic acid 40 mg/kg on day 10	Retinoic acid 40 mg/kg on day 11
+/- (<i>Pdn</i> /+ and/or +/+)			
Forelimb			
No. of specimens observed	134	93	92
Metacarpals	5.0±0.00	4.9±0.27**	4.8±0.43***
Proximal phalanges	5.0±0.09	5.0±0.27	4.8±0.45***
Middle phalanges	4.0±0.00	3.9±0.37*	3.8±0.43***
Distal phalanges	5.0±0.21	5.3±0.55***	5.3±0.59***
Hindlimb			
No. of specimens observed	134	91	92
Metatarsals	5.0±0.09	5.0±0.15	4.8±0.48***
Proximal phalanges	5.0±0.09	5.0±0.15	4.8±0.51***
Middle phalanges	4.0±0.00	3.9±0.25**	3.7±0.51***
Distal phalanges	5.6±0.49	5.1±0.35***	5.0±0.60***
<i>Pdn/Pdn</i>			
Forelimb			
No. of specimens observed	44	34	17
Metacarpals	7.0±0.63	7.1±0.81	7.2±0.53
Proximal phalanges	7.2±0.68	7.3±0.79	7.4±0.70
Middle phalanges	6.7±0.76	6.8±0.59	6.8±0.95
Distal phalanges	8.0±1.05	8.3±0.91	8.1±0.75
Hindlimb			
No. of specimens observed	42	34	16
Metatarsals	6.0±0.38	5.9±0.61	5.9±0.50
Proximal phalanges	6.1±0.45	5.9±0.65	5.9±0.57
Middle phalanges	5.8±0.80	5.7±0.71	5.5±0.82
Distal phalanges	6.5±0.74	6.6±0.78	6.2±0.83

Values are means ± S.D.

*, **, *** : Significantly different from vehicle control by Wilcoxon's rank-sum test ($P < 0.05, 0.01, 0.001$).

On the other hand, in *Pdn/Pdn* fetuses, RA treatment on day 11 induced the shortening of all stylopodium and zygopodium, but this effect seemed to be milder in *Pdn/Pdn* than +/- . Than is, stylopodium and zygopodium in *Pdn/Pdn* were longer than those in +/- (e.g. the length of fibula : 1.66 mm in *Pdn/Pdn* versus 0.88 mm in +/-).

From these results, we focused on the differences between genotypes in the shortening of stylopodium and zygopodium induced by RA treatment in the present experiment. Figs. 1 and 2 show bone- and cartilage-stained specimens of

the fore- and hindlimbs of fetuses on day 18 of gestation from vehicle control or RA-treated groups. In the vehicle control group, there were no differences in the lengths of stylopodium and zygopodium between +/- (Photo. 1A, C) and *Pdn/Pdn* (Photo. 1B, D) fetuses. However, in the RA-treated group, stylopodium and zygopodium, especially ulna and fibula, were severely shortened in +/- fetuses (Photo. 2A, C), and milder effects were observed in those of the *Pdn/Pdn* genotype (Photo. 2B, D). Two-way ANOVA was used to analyze the interaction between genotype and RA treatment (Table 5).

Table 3. Malformations of stylopodium and zygapodium in *Pdn* mice treated with all-*trans*-retinoic acid.

	Vehicle control olive oil 10 ml/kg on day 11	Retinoic acid 40 mg/kg on day 10	Retinoic acid 40 mg/kg on day 11
<i>+/- (Pdn/+ and/or +/+)</i>			
No. of specimens observed	134	97	92
Deformed humerus	0	42(43.3%)*	86(93.5%)*
Deformed radius	1(0.7%)	42(43.3%)*	86(93.5%)*
Deformed ulna	0	42(43.3%)*	78(84.8%)*
Humerus-radius synostosis	0	25(25.8%)*	29(31.5%)*
Humerus-ulna synostosis	0	4(4.1%)*	9(9.8%)*
Deformed femur	0	18(18.6%)*	70(76.1%)*
Deformed tibia	0	20(20.6%)*	72(98.3%)*
Deformed fibula	0	4(4.1%)*	45(48.9%)*
Absence of fibula	0	16(16.5%)*	31(33.7%)*
Femur-tibia synostosis	0	0	14(15.2%)*
<i>Pdn/Pdn</i>			
No. of specimens observed	44	34	18
Deformed humerus	0	6(17.6%)*	13(72.2%)*
Deformed radius	0	6(17.6%)*	12(66.7%)*
Deformed ulna	0	6(17.6%)*	13(72.2%)*
Humerus-radius synostosis	0	2(5.9%)	2(11.1%)
Humerus-ulna synostosis	0	2(5.9%)	2(11.1%)
Deformed femur	0	2(5.9%)	8(44.4%)*
Deformed tibia	24(54.5%)	7(20.6%)*	12(66.7%)*
Deformed fibula	0	1(2.9%)	10(55.6%)*
Absence of fibula	0	2(5.9%)	0
Femur-tibia synostosis	0	0	0

*, **, *** : Significantly different from vehicle control by Fisher's exact test ($P < 0.05, 0.01, 0.001$).

In both RA-treated groups, an interaction was seen in all stylopodium and zygapodium. From these analyses, it was confirmed that RA treatment induced milder effects in *Pdn/Pdn* than in *+/-* fetuses.

Other bone malformations : In *+/-* fetuses, the incidence of rib abnormalities was increased following RA treatment on day 11 of gestation, and increased rates of sternal cleft and coccygeal abnormalities were observed following RA treatment on day 10 of gestation.

In *Pdn/Pdn* fetuses, the incidence of coccygeal abnormalities was increased following RA treatment on day 10 of gestation, and sternal cleft was observed in all fetuses including those in the vehicle control group.

The incidence of rib abnormalities was lower in *Pdn/Pdn* than *+/-* fetuses in the group treated on day 11 of gestation. Fused sacral vertebrae and ischeal defect were observed in *+/-* fetuses treated with RA on both days.

Cartilage staining of the limb plates : In the cartilage of forelimbs on day 13 of gestation from the group treated with RA on day 11, stylopodium and zygapodium of *Pdn/Pdn* embryos, especially the ulna, were longer than those of *+/-* embryos (Photo. 3A, B). Milder effects were already observed in early chondrogenesis in *Pdn/Pdn* than *+/-* embryos on day 13 of gestation.

Immunohistochemical staining of forelimb plate : Apoptotic cell death was observed very sparsely in the limb mesenchyme immunohis-

Table 4. Lengths of stylopodium and zygapodium in *Pdn* mice treated with all-*trans*-retinoic acid.

	Vehicle control olive oil 10 ml/kg on day 11	Retinoic acid 40 mg/kg on day 10	Retinoic acid 40 mg/kg on day 11
<i>+/- (Pdn/+ and/or +/+)</i>			
Humerus	3.95 ± 0.184 (134)	3.56 ± 0.382*** (71)	2.77 ± 0.403*** (61)
Radius	3.18 ± 0.171 (134)	2.95 ± 0.305*** (72)	1.65 ± 0.487*** (63)
Ulna	4.09 ± 0.196 (134)	3.06 ± 0.932*** (93)	2.36 ± 0.509*** (83)
Femur	3.48 ± 0.173 (134)	3.36 ± 0.417** (97)	2.40 ± 0.530*** (76)
Tibia	3.47 ± 0.187 (134)	3.22 ± 0.592*** (93)	2.06 ± 0.742*** (77)
Fibula	3.30 ± 0.182 (134)	2.61 ± 1.249*** (97)	0.88 ± 0.981*** (92)
<i>Pdn/Pdn</i>			
Humerus	3.88 ± 0.266 (44)	3.79 ± 0.297 (32)	2.99 ± 0.290*** (14)
Radius	3.00 ± 0.244 (44)	2.94 ± 0.340 (32)	2.02 ± 0.516*** (16)
Ulna	4.02 ± 0.246 (44)	3.57 ± 0.628*** (32)	2.95 ± 0.625*** (16)
Femur	3.42 ± 0.211 (44)	3.45 ± 0.357 (34)	2.86 ± 0.373*** (16)
Tibia	3.02 ± 0.457 (44)	3.13 ± 0.527 (34)	2.19 ± 0.604*** (18)
Fibula	3.13 ± 0.264 (44)	2.97 ± 0.819 (34)	1.66 ± 0.968*** (18)

Values are means ± S.D. in mm.

() : No. of bones measured.

, * : Significantly different from vehicle control by Student's *t*-test ($P < 0.01$, 0.001).

tochemically at 12 hr after treatment with olive oil on day 11 of gestation (Photo. 4A). In contrast, many apoptotic cells were observed in the limb mesenchyme treated with RA on day 11 of gestation (Photo. 4B).

DISCUSSION

Kochhar (1977) suggested that mesomelia induced by retinoic acid was a result of specific effects on chondrogenesis and that retinoic acid

was cytotoxic only for cells of the chondrogenic lineage. Another possible mechanism for the teratogenicity of retinoic acid is the alteration of programmed cell death pattern (Sulik and Dehart, 1988; Alles and Sulik, 1989).

It has been demonstrated that programmed cell death in the limb bud and foot-plate plays an important role in normal limb morphogenesis (Scott *et al.*, 1977; Milaire and Rooze, 1983; Naruse and Kameyama, 1986). Milaire (1967) described the programmed cell death in the

Photo. 1. Bone- and cartilage-stained specimens of the fore- and hindlimbs of *Pdn* mouse fetuses on day 18 of gestation (vehicle control group).

A: Forelimb of *+/+* fetus treated with 10 ml/kg of olive oil on day 11 of gestation.

B: Forelimb of *Pdn/Pdn* fetus treated with 10 ml/kg of olive oil on day 11 of gestation.

C: Hindlimb of *Pdn/+* fetus treated with 10 ml/kg of olive oil on day 11 of gestation.

D: Hindlimb of *Pdn/Pdn* fetus treated with 10 ml/kg of olive oil on day 11 of gestation.

There were no significant differences in the lengths of stylopodium and zygopodium between *Pdn/Pdn* and *+/-* (*Pdn/+* and/or *+/+*) fetuses.

Photo. 2. Bone- and cartilage-stained specimens of the fore- and hindlimbs of *Pdn* mouse fetuses on day 18 of gestation (all-*trans*-retinoic acid-treated group).

A : Forelimb of *Pdn*/+ or +/+ fetus treated with 40 mg/kg of retinoic acid on day 11 of gestation.

B : Forelimb of *Pdn/Pdn* fetus treated with 40 mg/kg of retinoic acid on day 11 of gestation.

C : Hindlimb of *Pdn*/+ or +/+ fetus treated with 40 mg/kg of retinoic acid on day 11 of gestation.

D : Hindlimb of *Pdn/Pdn* fetus treated with 40 mg/kg of retinoic acid on day 11 of gestation.

Stylopodium and zygopodium, especially ulna (arrows) and fibula (triangles), were shortened by the retinoic acid treatment. Milder effects were observed in *Pdn/Pdn* than +/- (*Pdn*/+ and/or +/+) fetuses.

Table 5. Results of two-way ANOVA of the lengths of stylopodium and zygapodium in *Pdn* mice treated with all-*trans*-retinoic acid.

Vehicle control group versus that treated with retinoic acid on day 10

Variable	Main effect		Two-way interaction
	Genotype	Treatment	Genotype by treatment
Humerus	<0.05	<0.001	<0.001
Radius	<0.01	<0.001	<0.01
Ulna	<0.01	<0.001	<0.001
Femur	NS	NS	<0.05
Tibia	<0.001	NS	<0.01
Fibula	NS	<0.001	<0.01

NS : not significant

Vehicle control group versus that treated with retinoic acid on day 11

Variable	Main effect		Two-way interaction
	Genotype	Treatment	Genotype by treatment
Humerus	NS	<0.001	<0.01
Radius	NS	<0.001	<0.001
Ulna	<0.001	<0.001	<0.001
Femur	<0.001	<0.001	<0.001
Tibia	<0.05	<0.001	<0.001
Fibula	<0.01	<0.001	<0.001

NS : not significant

central mesenchyme of the mouse limb on day 11 and in the area between the presumptive zygapodia on day 12 of gestation. Excessive cell death in the programmed cell death area was proposed as the pathogenic mechanism of limb malformations evoked by RA treatment (Alles and Sulik, 1989). Increases in the level of cell death caused by 13-*cis*-retinoic acid have been reported in the programmed cell death zones (Sulik and Dehart, 1988). Excessive cell death induced by RA would interfere with the process of mesenchymal condensation, causing limb anomalies with short, misshapen, or missing bones.

RA treatment on day 10 or 11 or gestation induced the shortening of stylopodium and zygapodium in *Pdn* mice. Differences in the frequency of limb anomalies and the lengths of stylopodium and zygapodium were observed between *Pdn/Pdn* and +/- fetuses; RA exposure exhibited milder effects in *Pdn/Pdn* than +/- fetuses,

and interactions between RA treatment and genotype were observed in the length of stylopodium and zygapodium. Many apoptotic cells were observed in the limb mesenchyme at 12 hr after treatment with RA. From these results, it was considered that excessive cell death induced by RA in the central region of the limb mesenchyme might be prevented in *Pdn/Pdn* fetuses.

Pdn/Pdn embryos exhibit abnormal programmed cell death patterns such as the abolishment of the deep preaxial mesodermal cell death zone (*foyer primaire préaxial*) leading to the preaxial polydactyly (Naruse and Kameyama, 1982; Naruse and Kameyama, 1986). From this finding, we have speculated that *Pdn* gene expression may have a role in programmed cell death during embryogenesis. Thus, we speculated that excessive cell death induced by RA might be prevented by *Pdn* gene expression, though we need further investigations.

Though RA induced many hypogenetic

Photo. 3. Cartilage-stained specimens of the forelimb of *Pdn* mouse embryos on day 13 of gestation.
 A : Forelimb of *Pdn*/+ or +/+ embryo treated with 40 mg/kg of retinoic acid on day 11 of gestation.
 B : Forelimb of *Pdn*/*Pdn* embryo treated with 40 mg/kg of retinoic acid on day 11 of gestation.
 The length of the ulna (arrows) differed between +/- (*Pdn*/+ and/or +/+) and *Pdn*/*Pdn* embryos already on day 13 of gestation.

Photo. 4. Immunohistochemical staining using anti-single-stranded DNA antibody in the forelimb mesenchyme on day 11.5 of gestation. (bar=14 μ m)
 A : Forelimb mesenchyme in the fetus treated with 10 ml/kg of olive oil on day 11 of gestation.
 B : Forelimb mesenchyme in the fetus treated with 40 mg/kg of retinoic acid on day 11 of gestation.
 Many apoptotic cells (arrows) were induced at 12 hr after the retinoic acid treatment.

anomalies in the limb, the increase of the number of distal phalanges in the forelimbs was observed in +/- fetuses in both RA-treated groups. That is, one extra digit was induced postaxially. It is known that RA induce not only oligodactyly but also postaxial polydactyly (Cusic and Dagg, 1985; Bynum, 1991). However, the mechanism of this phenomenon is considered not to be clear at present.

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