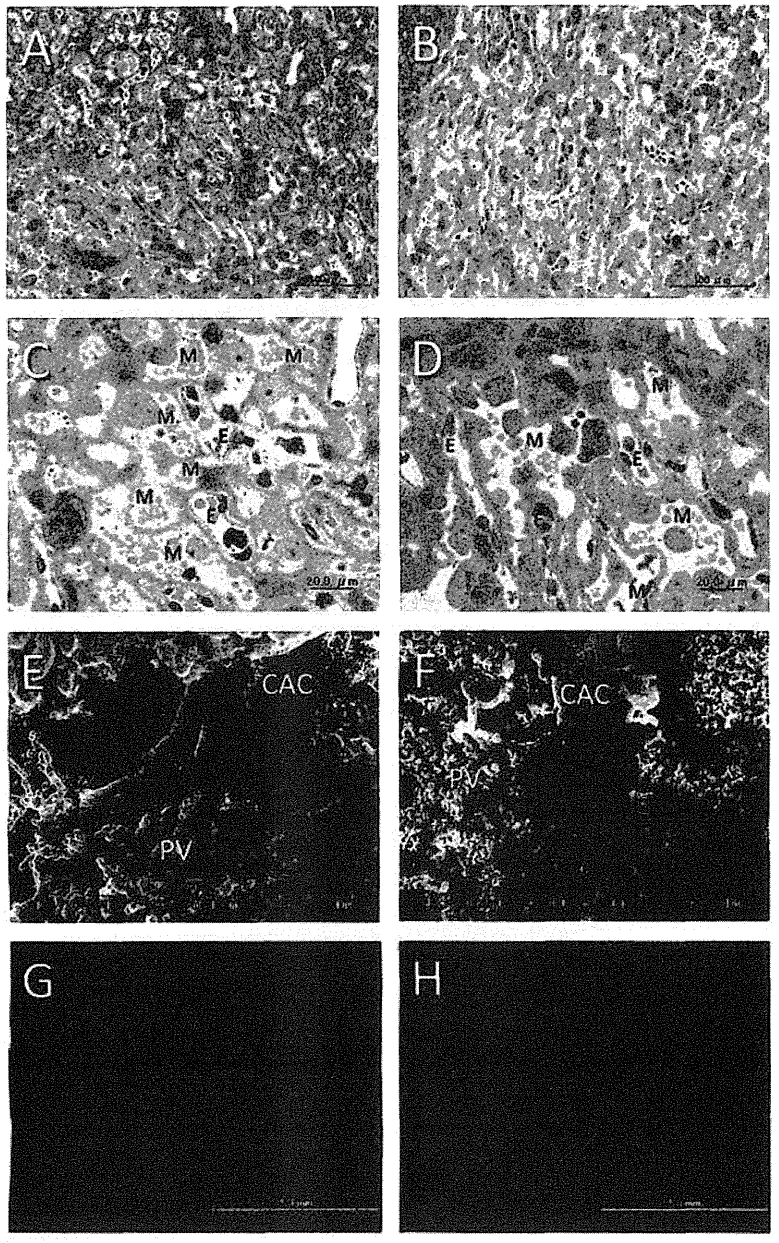

Colour

Fig. 1 Histological findings of placenta from dams on day 13 of gestation. (A,B) The labyrinth zone (LZ) of the placentas of a control dam (A) and a dam exposed to diethylstilbestrol (DES) at 10 μg/kg per day on days 4 through 8 of gestation showing impaired villous network (B). Toluidine blue staining. (C,D) Higher magnification of A, showing the maternal blood space (M) and the embryonic capillary (E) and higher magnification of B, showing the dilatation of M. (E,F) Ultrastructure of the LZ of the placenta. The plexus vasculosus (PV) in the LZ of the DES group (F) was thinner than that of the control group (E), although the branching structure of the DES group was well developed similar to that in the controls. There were no distinct differences in development of the central arterial canal (CAC) between the DES group and the control group. (G,H) Immunolocalization of GLUT-1 in the LZ of placentas. Note that GLUT-1 expression is abundant in the control LZ (G), and decreased in the LZ of the placenta from a dam exposed to DES (H).

pregnancy is GLUT-1, and immunohistochemically, the rat placental LZ is rich in GLUT-1 (Tanaka et al. 1994). GLUT-1 is localized at both maternal and fetal sides of the plasma membranes of syncytiotrophoblasts. Abundant GLUT-1 in such critical plasma membranes of the barrier may be crucial to the transplacental transfer of glucose in human and rodent placentas.

Blood glucose levels of dams and fetuses

We examined the changes in blood glucose levels because development of the LZ including the expression of GLUT-1 protein was impaired by early to middle gestational exposure to DES. Glucose is a major nutrient that passes through the placenta from the mother to the growing embryo. The blood glucose levels in the embryos of the DES group (103.9 ± 14.1 mg/dL) were significantly ($P < 0.01$) decreased compared with those in the control embryos (120.0 ± 19.1 mg/dL), whereas the glucose levels in dams of the DES group (157.8 ± 13.5 mg/dL) were comparable to those in the controls (152.7 ± 14.0 mg/dL). Thus, it is reasonable to suggest that underdevelopment of the LZ may be associated with a reduction in the capacity to transport glucose.

CONCLUSION

Oral exposure to DES during early to middle gestation affects development of the LZ in the mouse placenta, and impairment of the LZ may be related to a decrease in GLUT-1 expression in the LZ, and the reduction of blood glucose levels in the embryos. Taking these factors together, it is reasonable to suggest that the developmental disturbance of the placenta induced by DES administration may be associated with embryolethality of DES in mice.

CONFLICT OF INTEREST

The authors declare that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriately influence, or be perceived to influence, the work.

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Newly developed mouse newborn behavioral testing method for evaluating the risk of neurotoxicity of environmental toxicants

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ABSTRACT: Although there have been a vast number of behavioral toxicology studies carried out on adult mice and rats, there have been few neurobehavioral studies utilizing their newborn animals. Thus, we developed a mouse newborn behavioral testing method for evaluating the risk of neurotoxicity of chemicals, by means of determining the newborn's activity using the tare function of an analytical balance. The unstable weighing values resulting from movement of the newborn on the balance recorded by a personal computer every 0.1 s, and the total activities of a newborn from the start time of weighing to individual times of evaluation were calculated. In addition, we confirmed the usefulness of our method by determining the activity of mouse newborns with microcephaly induced by prenatal exposure to a neurotoxicant, methylnitrosourea. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: behavioral test; mouse newborn; neurotoxicity; activity; electric balance

Introduction

The estimated frequencies of neurodevelopmental disorders, including autism, dyslexia, attention-deficit hyperactivity disorder, decreased intelligence and mental retardation, in children are relatively high, i.e. around 12% (Hass, 2006). The central nervous system appears to be especially susceptible to toxic insults during development and there is evidence that functional changes can be induced at a lower exposure level that those resulting in toxicity in adults (Kimmel, 1988; Bearer, 2000). The tragic methyl mercury poisoning of humans in Minamata Bay in the 1950s led to animal research on the general neurotoxicity and prenatal effects of methyl mercury. The results showed that the embryo/fetus was more susceptible to exposure than adult animals, and that exposed animals exhibited similar adverse effects as those reported in humans (Burbacher *et al.*, 1990).

Some of the developmental neurotoxicants are structural teratogens as well, but the behavioral dysfunctions may be more serious than the structural defects. Investigations demonstrate that, under certain circumstances, neurobehavioral dysfunction in children can be detected at a lower dose than congenital malformations (ethanol) or in the complete absence of malformations (polychlorinated biphenyl) (Francis *et al.*, 1990).

Although there have been a vast number of animal toxicology studies carried out on pregnant animals including embryos/fetuses and mature animals, there is a paucity of reports on animal toxicology studies utilizing newborn, infant and juvenile animals. Thus, we developed a mouse newborn neurobehavioral testing method; it involves quantitative determination of a newborn animal's activity automatically using the tare function of an analytical balance, in order to evaluate the risks of neurotoxicities of various environmental toxicants including low-dose endocrine-disrupting chemicals.

Materials and Methods

Animals

ICR mice at 8 weeks old purchased from CLEA (Osaka, Japan) were used after acclimation for 2 weeks. The experimental protocols were approved by the Animal Care and Use Committee of Kinki University. Mice were kept under SPF conditions and housed in polycarbonate cages in a room in which the temperature (23 ± 1 °C), humidity (50 ± 5%), and light cycle (12:12 h light–dark cycle; lights on at 7 a.m.) were maintained. Mice were allowed food (Certified Rodent Chow CE-2, CLEA, Osaka, Japan) and drinking water *ad libitum*. Ten-week-old virgin female mice were cohabited for 2 h (7–9 a.m.) on a 1:1 basis with males aged 11 weeks or older. Females were checked for the presence of a vaginal plug immediately thereafter, and 8 a.m. was defined as the time of conception if a plug was found. Pregnant mice were allowed to give birth and nurse their pups until postnatal day (PND) 5. In the morning on PND 1, the number of pups in a litter was adjusted to four males and four females. Pups were weighed on PNDs 1 and 5,

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the number of pups in each litter during the lactation period was recorded, and viability on PND 5 after adjustment of the number was determined.

Determination of Newborn Activity Using an Electric Balance

An electric balance [Tuning-fork analytical balance, HTR-80, Shinko Denshi Co. Ltd, Tokyo, capacity 80 g, readability 0.0001 g, repeatability (σ) 0.0001 g, interface RS232C, D-SUB9P] on a shock-proof stage was used to evaluate the absolute values obtained from the range of fluctuations between weighing values resulting from the movement (walking/righting, tremor) of newborns on PND 1, 2 or 3 at 10 a.m., 12, 2, 4 and 6 p.m.. The absolute value was defined as the activity of a newborn, and the total activity of a newborn was the sum total of the absolute values during the period evaluated. The unstable weighing values obtained by the movement of the newborn in the plastic dish (94/16, Greiner Bio-One GmbH., Austria) on the pan of the balance were recorded by a personal computer every 0.1 s via WinCT (Windows Communication Tools) software (version 3.00, A&D Company Ltd, Tokyo, Japan). The possibility of reflection was checked using a fixed weight (5 g) on the balance for 3 min before the measurement of newborn activity in order to ensure that the weights of newborns on the balance did not reflect drift in the value of the balance. The room in which the measurement was carried out was maintained under the same experimental conditions, such as temperature and humidity, as the animal room.

Table 1 shows representative data for a mouse newborn weight sent from the balance every 0.1 s, and the method of quantitative determination of newborn activity by Microsoft Excel 2007. *B* shows the real time and the time after the start of measurement; *C* is newborn weight (weighing value) that was sent from a balance every 0.1 s; *D* shows the difference between the weighing value and that 0.1 s beforehand ($D = Cn - Cn - 1$); *E* is the absolute value of *D* ($E = |D|$).

Subsequently, in order to determine the total activity (*F*) of a newborn from the start time of weighing to an individual time (*B*), each *E* value was added up ($En + Fn - 1$; Fig. 1(B)), and total activities for 6 min ($0.1\ s \times 600 \times 6$) were determined and are shown in Fig. 1(A).

Exposure of Pregnant Dams to MNU and Determination of MNU-Treated Newborn Activities by the Newly Developed Testing Method

N-Methyl-*N*-nitrosourea (MNU) was purchased from Sigma Chemical (St Louis, MO, USA), dissolved in distilled water, and administered intraperitoneally on day 13 of gestation because this embryonic day is a part of the organogenesis period of the mouse cerebral cortex and the peak of neurogenesis in the primordium (Komada *et al.*, 2010). Dose solution was prepared prior to dosing. Five mice administered distilled water (10 ml kg^{-1} body weight) were used as controls. Five mice were administered MNU at 10 mg kg^{-1}. Administration occurred at a defined time (12:00–12:10 p.m.), and pregnant mice were allowed to give birth and nurse their newborns until PND 5. Subsequently, the activities of newborns were determined for 6 min from 12 p.m. on PND 1. The number of pups used in this study was 20 males and 20 females per group (four males and four females per litter).

For the effects of MNU, the data were analyzed via two-way analysis of variance (ANOVA) with treatment and gender (male vs female) as the factors. Whether the repeated measure ANOVA detected significant interactions or not, one-way ANOVA was followed by tests for simple main effects, and detailed multiple comparisons were made with Tukey's honestly significant difference *post hoc* tests, given corresponding significant *F*-values. Statistical analyses were performed using StatView (version 5.0; SAS Institute, Cary, NC, USA). All data used the litter average as the statistical unit, and statistical significance was assumed for probability levels of 0.05 or less.

Results and Discussion

Comparison of Activity between the Newborns on Different PNDs or between the Newborns at Different Starting Times of Measurement in a Day

The activity was determined on PND 1, 2 or 3 using the intact mouse newborns. The activities of individual newborns on PND 2 and 3 showed wide variation compared with those on PND 1. In addition, activities of individual newborns determined at 12 p.m. on any PND did not vary widely from those at 10 a.m., 2, 4 and 6 p.m. on any PND (data not shown). Using these results obtained from the intact newborns, the activities were compared between the control and MNU-treated newborns at 12 p.m. on PND 1.

The activities of mouse newborns analyzed in the present study include relatively large movements, such as righting and walking, and small movements, such as tremors specific to newborns. Of particular importance is evaluation of these types of movement separately. In the testing method developed in this study, the weighing values for the large movements of the newborns showed large absolute values (Table 1, column *E*). We defined the large movements (righting and walking) of the newborns as activities showing an absolute value (*E*) of 0.0002 or more (Table 2). Bold entries (*E*53–59) and nonbold entries (*E*50–52, *E*60–64) in this table show the large movements and small movements, respectively, of newborns. An integration of absolute values (*E*) showing 0.0002 or more for 6 min is shown in Fig. 2. The gray area in this figure shows the large movements of newborn. Of particular interest is a comparison of the changing patterns, such as this figure comparing the control group and a chemical-treated group for evaluation or identification of various kinds of neurotoxicants that exist in the environment.

How to determine the newborn activity separately according to the types of movement will be described in elsewhere. Namely, the precise distinction between the absolute values (*E*60, 61) of 0.0001 contained in the weighing values of the large movement (Table 2) and the absolute values (*E*15) of 0.0001 in the small movement (Table 1) is needed for further analyses of the rodent newborn activity in the test method.

Maternal and Developmental Effects of Prenatal MNU Exposure

No adverse effects on the general conditions, such as decreased body weight gain, death, moribund state, loss of spontaneous activity, crouching and piloerection in maternal animals were observed in the MNU-treated group. All of the pregnant mice delivered normally by 12:00 p.m. on day 19 of gestation. No significant differences in the number and the viability of newborns on PND 1 were found between the MNU-treated

Table 1. How to determine the total activity of individual newborn

A	*B*		*C*	*D*	*E*	*F*
No.	Real time	Time after start (s)	Weighting value of pup[a] (g)	Difference between the weighing value and that 0.1 s beforehand ($Dn = Cn - Cn-1$)	Absolute value ($En = \|Dn\|$)	Total activity[b] ($Fn = En + Fn-1$)
1	12:01:02			Before start of determination		
2	12:01:02			Before start of determination		
3	12:01:03	0	$C3 = 2.0227$	$D3 = +0$ ($C3 - C2 = 2.0227 - 2.0227$)	$E3 = 0.0000$	$F3 = 0.0000$ ($E3 + F2 = 0.0000 + 0.0000$)
4	12:01:03	0.1	$C4 = 2.0231$	$D4 = +0.0004$ ($C4 - C3 = 2.231 - 2.0227$)	$E4 = 0.0004$	$F4 = 0.0004$ ($E4 + F3 = 0.0000 + 0.0004$)
5	12:01:03	0.2	$C5 = 2.0233$	$D5 = +0.0002$ ($C5 - C4 = 2.0233 - 2.0231$)	$E5 = 0.0002$	$F5 = 0.0006$ ($E5 + F4 = 0.0004 + 0.0002$)
6	12:01:03	0.3	$C6 = 2.0233$	$D6 = +0$ ($C6 - C5 = 2.0233 - 2.0233$)	$E6 = 0.0000$	$F6 = 0.0006$ ($E6 + F5 = 0.0006 + 0.0000$)
7	12:01:03	0.4	$C7 = 2.0231$	$D7 = -0.0002$ ($C7 - C6 = 2.0231 - 2.0233$)	$E7 = 0.0002$	$F7 = 0.0008$ ($E7 + F6 = 0.0006 + 0.0002$)
8	12:01:03	0.5	$C8 = 2.0228$	$D8 = -0.0003$ ($C8 - C7 = 2.0228 - 2.0231$)	$E8 = 0.0003$	$F8 = 0.0011$ ($E8 + F7 = 0.0008 + 0.0003$)
9	12:01:03	0.6	$C9 = 2.0225$	$D9 = -0.0003$ ($C9 - C8 = 2.0225 - 2.0228$)	$E9 = 0.0003$	$F9 = 0.0014$ ($E9 + F8 = 0.0011 + 0.0003$)
10	12:01:03	0.7	$C10 = 2.0222$	$D10 = -0.0003$ ($C10 - C9 = 2.0222 - 2.0225$)	$E10 = 0.0003$	$F10 = 0.0017$ ($E10 + F9 = 0.0014 + 0.0003$)
11	12:01:03	0.8	$C11 = 2.0219$	$D11 = -0.0003$ ($C11 - C10 = 2.0219 - 2.0222$)	$E11 = 0.0003$	$F11 = 0.0020$ ($E11 + F10 = 0.0017 + 0.0003$)
12	12:01:03	0.9	$C12 = 2.0217$	$D12 = -0.0002$ ($C12 - C11 = 2.0217 - 2.0219$)	$E12 = 0.0002$	$F12 = 0.0022$ ($E12 + F11 = 0.0020 + 0.0002$)
13	12:01:04	1.0	$C13 = 2.0216$	$D13 = -0.0001$ ($C13 - C12 = 2.0216 - 2.0217$)	$E13 = 0.0001$	$F13 = 0.0023$ ($E13 + F12 = 0.0022 + 0.0001$)
14	12:01:04	1.1	$C14 = 2.0217$	$D14 = +0.0001$ ($C14 - C13 = 2.0217 - 2.0216$)	$E14 = 0.0001$	$F14 = 0.0024$ ($E14 + F13 = 0.0023 + 0.0001$)
15	12:01:04	1.2	$C15 = 2.0218$	$D15 = +0.0001$ ($C15 - C14 = 2.0218 - 2.0217$)	$E15 = 0.0001$	$F15 = 0.0025$ ($E15 + F14 = 0.0024 + 0.0001$)

[a]Weighing value of pup is a mouse newborn weight sent from the balance every 0.1 s.
[b]In order to determine the total activity (F) of a newborn from the start time of weighing to an individual time (B), each absolute value (E) was added up ($En + Fn-1$).

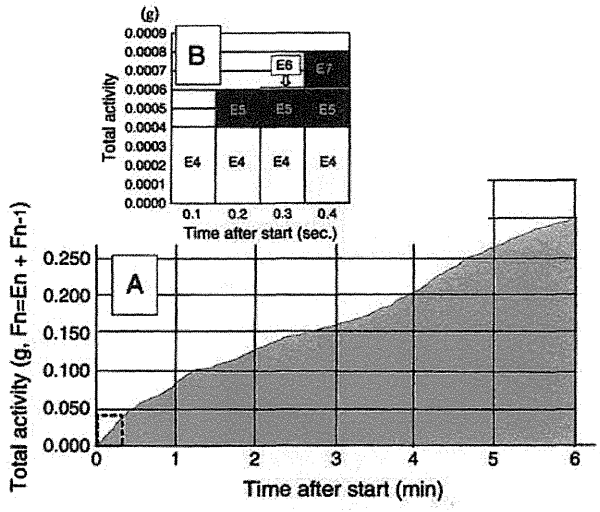

Figure 1. Total activities of intact newborn for 6 min (A) and the magnification (B, 0.4 s; see Table 1) of the area indicated by a broken line in (A).

Table 2. Integration of absolute values showing large movements of newborn

A	*E*	*F*
No.	Absolute value ($En = \|Dn\|$)	Total activity ($Fn = En + Fn-1$)
50	$E50 = 0.0001$	
51	$E51 = 0.0001$	
52	$E52 = 0.0000$	
53	**$E53 = 0.0003$**	**$F53 = 0.0003$**
54	**$E54 = 0.0005$**	**$F54 = 0.0008$**
55	**$E55 = 0.0007$**	**$F55 = 0.0015$**
56	**$E56 = 0.0011$**	**$F56 = 0.0026$**
57	**$E57 = 0.0008$**	**$F57 = 0.0034$**
58	**$E58 = 0.0004$**	**$F58 = 0.0038$**
59	**$E59 = 0.0002$**	**$F59 = 0.0040$**
60	$E60 = 0.0001$	
61	$E61 = 0.0001$	
62	$E62 = 0.0000$	
63	$E63 = 0.0001$	
64	$E64 = 0.0001$	

The large movements of the newborns were defined as activities showing an absolute value (E) of 0.0002 or more. Bold and nonbold entries show the large movements and small movements, respectively, of newborns.

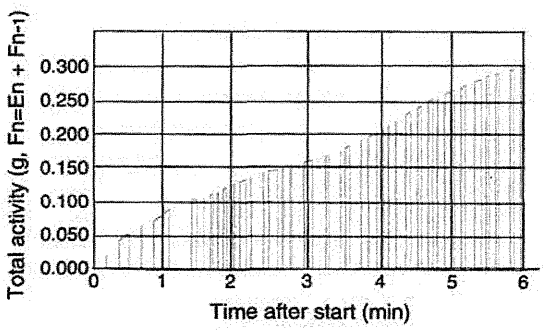

Figure 2. An integration of only absolute values showing large movements of newborn.

group and the control group. In addition, viability on PND 5 in the MNU-treated group was comparable to that in the controls. On PND 5, 20 male offspring from five litters in each group were weighed, and we measured the length of the interhemispheric fissure and the length of rostral-caudal telencephalon to determine (the length of the interhemispheric fissure/the length of rostral-caudal telencephalon) ratio. The ratio in the group treated with MNU was significantly smaller than that in the controls. The body weight of male and female offspring treated with MNU was comparable to that of the control offspring when evaluated on PNDs 1 and 5. In addition, male and female pups had comparable body weights in both groups (data not shown).

In our previous studies, we demonstrated that embryos treated with MNU at 10 mg kg^{-1} on day 13 of gestation show thinning of the cortical plate and subventricular zone/ventricular zone in the dorsal telencephalon, and that embryonic day 13 is a critical period for microcephaly in mice (Fujiyama *et al.*, 2007; Komada *et al.*, 2010). Thus, we investigated the activity of PND 1 newborns exposed to MNU on day 13 of gestation in order to elucidate the scientific utilization of the developed behavioral testing method for evaluating the risk of neurotoxicity of MNU.

Activity of Newborns Exposed Prenatally to MNU

Figure 3 shows representative patterns concerning the activities for 6 min of male newborns exposed prenatally to MNU at 10 mg kg^{-1} and control newborns on PND 1. The shape, slope and area under the curve in each graph were clearly different between the MNU-treated group and the control group. Subsequently, the mean total activities of male newborns from 0 to 2 min, from 2 to 4 min and from 4 to 6 min at 12 p.m. on PND 1 were determined in the MNU-treated group and the control group according to the methods shown in Table 1 and Fig. 1(B). The weight data collected from 0 to 2, from 2 to 4 and from 4 to 6 min were analyzed with an approach that take into account the multiple measures from each animal using a repeated measures ANOVA. There were significant differences in the activity and measurement time in both sexes [males, F (2, 27) = 6.36, P = 0.0093; females, F (2, 27) = 4.24, P = 0.0334]. Activities for the first two time blocks in the MNU-treated group showed no significant difference from those in the controls, and those from 4 to 6 min in the group treated with MNU were significantly higher than those in the controls. The ANOVA for males and females revealed main effects of treatment [F (1, 8) = 17.47, P = 0.0031, and F (1, 8) = 22.94, P = 0.0014, respectively]. No significant differences were detected between those from 0 to 2 and from 2 to 4 min in the group treated with MNU and those in the controls in both sexes. The total activities for 6 min in the MNU-treated group were significantly higher than those in the control group in both sexes (Fig. 4). The ANOVA for males and females revealed main effects of treatment [F (1, 8) = 7.75, P = 0.0238, and F (1, 8) = 24.32, P = 0.0011, respectively]. In addition, the ANOVA revealed no effect of MNU on body weight in either males or females [F (1, 8) = 0.15, P = 0.7112, and F (1, 8) = 0.091, P = 0.7704, respectively].

There are numerous behavioral tests to evaluate learning and memory, and the activity of rats and mice with microcephaly induced by neurotoxicants using Biel maze and radial arm maze, shuttlebox and wheel cage (Akaike *et al.*, 1994). Ohta *et al.* (1997) demonstrated that rat offspring with microcephaly induced by prenatal MNU exposure exhibited increased wheel cage activity when evaluated at 6 and 9 weeks of age, suggesting hyperactivity in adult rats exposed *in utero* to a neurotoxicant. However, few neurobehavioral tests have been developed in

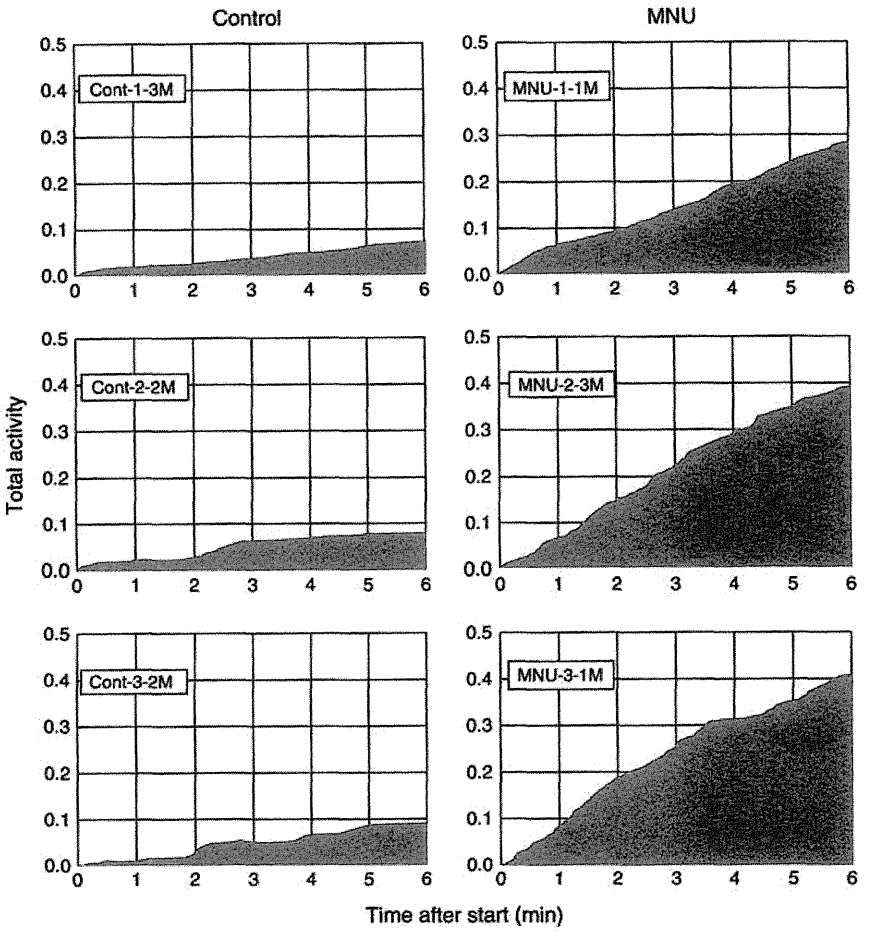

Figure 3. *Representative pattern of activities for 6 min of PND 1 male newborns from three dams each in the control group and the MNU-treated group.*

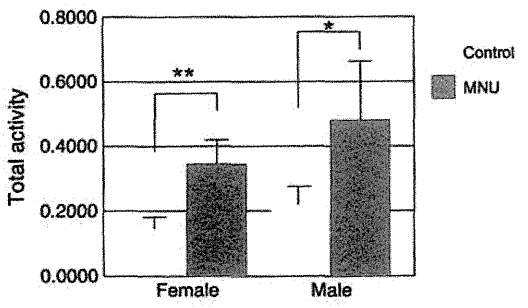

Figure 4. Mean total activities of PND 1 newborns from five dams each in the control group and the MNU-treated group. Vertical lines represent standard deviations. * Significantly different from the control, $P < 0.05$; ** significantly different from the control, $P < 0.01$.

order to evaluate the newborn behavior and activity. Hashimoto and Mizutani (1991) showed early neurobehavioral disorders in microcephalic rat offspring induced by prenatal exposure to MNU at 5 mg kg^{-1}. The microcephalic pups during the suckling period, PND 0–22, were retarded in terms of neurobehavioral ontogeny. They showed impaired performance, such as paired limb movement, clumsy locomotion or hyperreflexive reaction, without growth retardation. In their study, some behavioral disorders such as strengthened hyperactivity, increased susceptibility to seizure and deficit in rotarod performance were detected in the neonatal rats after PND 3. Neurobehavioral changes of newborns on PND 0 or 1 have not been detected in any developed behavioral testing methods. In the present study, we clearly showed the quantitative changes of neurobehavior of mouse newborns on PND 1.

Of particular importance is consideration of the limitations and potential pitfalls of the proposed technique. The measurement proposed in this study uses changes in weight on the balance as the measure of activity. The same degree of movement in mouse newborns of different weights (i.e. ages and gender) would produce different changes in weight detected on the balance. Thus, on the basis of changes in weight, this would suggest that newborns of different weights have different levels of activity when they actually have comparable levels of activity. The same amount of movement in PND 1 and PND 5 newborns would translate into different weight changes, suggesting different levels of activity. The consequence of this is that using changes in weight as proposed cannot be used directly to compare movement between animals of different sizes (e.g. males vs females, different ages).

In conclusion, we have developed a rodent newborn behavioral testing method using an analytical balance. This developed technique is a practical approach to solve one of the challenges in assessing early effects of neurotoxicants and it may provide a useful addition to neurobehavioral assessment in very young rodents. As for the advantages of our method, it is considered that there is a possibility of early detection of neurobehavioral abnormalities and early medical treatment by means of the

analyses of newborn activity by this test method, as well as the measurement of newborn activity without stress such as pain. As the next step of our neurobehavioral studies using this technique, the dose–response relationship between newborn activities and the doses of a neurotoxicant should be clarified. The new technique proposed is simple. If this approach is validated, it may have the potential to become widely used.

Acknowledgments

We thank all our colleagues, including M. Shirazaki, T. Inada and H. Irie, who contributed to the present study. This study was partly supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labour and Welfare.

Conflict of Interest

The authors declare that there are no conflicts of interest

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Research Article

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Developmental effects of oral exposure to diethylstilbestrol on mouse placenta

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ABSTRACT: Placental growth and function are of biological significance in that placental tissue promotes prenatal life and the maintenance of pregnancy. Exposure to synthetic estrogens causes embryonic mortality and placental growth restriction in mice. The aim of the present study was to examine the effects of diethylstilbestrol (DES) on placenta in mice. DES at 1, 5, 10 or 15 μg kg^{-1} day^{-1}, or 17β-estradiol (E_2) at 50 μg kg^{-1} day^{-1}, was administered orally to ICR mice on days 4 through to 8 of gestation. Expression of ERα, ERβ, ERRβ or ERRγ mRNA in the junctional or labyrinth zone of the placentas on day 13 was assessed using RT-PCR, as well as the embrynic mortality, embryonic and placental weight, histological changes of labyrinth and ultrastructural changes of the trophoblast giant cells (TGCs). Embryo mortalities in the DES 10 and 15 μg kg^{-1} day^{-1} groups were markedly increased. No significant changes in embryonic and placental weight were observed in any DES- or E_2-exposed groups. Expression of ERα mRNA in the junctional zone with male embryos in the 5 μg kg^{-1} day^{-1} group was significantly higher than that in the control, whereas expression was not determined in the 15 μg kg^{-1} day^{-1} group. Histological observation revealed that the placentas exposed to DES at 10 μg kg^{-1} day^{-1} lacked the developing labyrinth. Ultrastructural observation of the TGCs showed poor rough-surfaced endoplasmic reticulum in the DES 10 μg kg^{-1} day^{-1} group. The present data suggest that developmental changes induced by DES may be related to interference with the nutrition and oxygen exchange between mother and embryo or decreased protein synthesis, resulting in a high frequency of embryo mortality.

Keywords: diethylstilbestrol; placental development; trophoblast giant cells; labyrinth; rough-surfaced endoplasmic reticulum

Introduction

The placenta is the first organ to form during mammalian embryogenesis. Problems in its formation and function underlie many aspects of early pregnancy loss and pregnancy complications in humans. Trophoblast cells are the first lineage to form in the mammalian conceptus and contribute exclusively to the extraembryonic structures that form the placenta (Cross *et al.*, 1994), which is critical for the survival of mammalian embryos. After implantation, the mural trophectoderm differentiates into the primary trophoblast giant cells (TGCs). The development of TGCs is essential to embryo implantation and to the maintenance of pregnancy. The placenta undertakes many functions beyond simple exchange between the maternal and fetal environment. The mouse placenta is a highly tuned exchange bed with supporting cells involved in both structural and functional aspects of fetal–maternal exchange. The mouse and the human both have discoid hemochorial placentas, but the mouse placenta is divided into two morphologically and functionally distinct regions known as junctional (basal) and labyrinth zones. The junctional zone, which includes TGCs and spongiotrophoblast cells, is involved in the formation of hormones, angiogenic factors, vascular endothelial growth factor, tissue remodeling factors and urokinase-type plasminogen activator. The labyrinth zone, on the other hand, is the major site of feto–maternal exchange, the point of nutrient transfer between the maternal and fetal blood spaces (Rossant and Cross, 2001; Georgiades *et al.*, 2002; Malassiné *et al.*, 2003).

Diethylstilbestrol (DES) is a synthetic (stilbene) estrogen with an *in vivo* estrogenic potency similar to that of 17β-estradiol (E_2), and is structurally related to E_2. DES (daily dose of 5–125 mg during pregnancy, Coscrove *et al.*, 1977, Oradell, 1961) was widely used to prevent a threatened miscarriage from the 1940s to 1971. DES can be metabolized to conjugates (glucuronide and sulfate) and to numerous oxidative metabolites (reviewed by Metzler, 1981). The teratogenicity, reproductive toxicity and carcinogenicity of DES in humans are believed to be transduced via the classic estrogen receptors ERα and ERβ (Couse and Korach, 1999; Greco *et al.*, 1993). DES has also been shown to induce placental changes that include a reduction of the labyrinth zone and accumulation of TGCs in mid- and late gestation in mice (Scott and Adejokun, 1980). These effects are unlikely to involve the classic ERs as genetic ablation of both receptors, singly or in combination, did not reveal a role for these receptors in placentation (Couse and Korach, 1999). In our previous study, we demonstrated that decidual hypoplasia and subsequent placental hemorrhage cause fetal death as a result of the oral administration of DES during the early stage of pregnancy in mice (Nagao and Yoshimura, 2009).

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Pharmacological doses of estrogens cause high levels of embryonic mortality (Haddad and Ketchel, 1969), although administration later in pregnancy causes retarded fetal growth with less of an effect on survival (Kuhn *et al.*, 1982). In order for estrogens to exert their biological effects, they need to bind to the ER, which then undergoes a conformational change allowing it to interact with chromatin and to modulate transcription of target genes (Tsai and O'Malley, 1994). ERα and ERβ proteins are physiologically relevant; they bind estradiol with high affinity and activate transcription of estrogen-responsive reporter gene constructs expressed in mammalian cell lines (Kuiper *et al.*, 1996, 1997). Little information on the expression profile of ER mRNA in developing rodent placentas is available. The expressions of ER isoforms and variants have been studied in rat placenta on days 16, 19 and 21 of gestation, and it has been demonstrated that ERα and ERβ mRNA were detected from as early as day 16 of gestation (Al-Bader, 2006). In mouse placenta, the highest expressions of ERα and ERβ mRNA were determined on day 14 of gestation (Nagao *et al.*, unpublished data).

Classic endocrine studies have shown that steroid hormones are required for the maintenance of pregnancy and placental viability. The estrogen receptor-related receptor β (ERRβ) is an orphan member of the superfamily of nuclear hormone receptors (Giguère *et al.*, 1988; Knöfler *et al.*, 2001). The protein is homologous to the ER and binds the estrogen response element. Luo *et al.* (1997) reported that ERRβ has an important role in early placentation. Tremblay *et al.* (2001) demonstrated that DES promotes coactivator release from ERRβ and inhibits its transcriptional activity, and the treatment of trophoblast stem cells with DES led to their differentiation towards the polyploidy giant-cell lineage, and that DES-treated pregnant mice exhibited abnormal early placenta development associated with an overabundance of TGCs and an absence of diploid trophoblasts.

To address the adverse effects of oral DES administration on placentogenesis resulting in embryonic death, the present study was designed to examine the ultrastructural changes of the TGCs in the trophoblast layer between the spongiotrophoblast layer and the decidua basalis, and the expression of ER or ERR mRNA. In addition, the development of the labyrinth zone where most gaseous and nutrient exchange occurs was investigated.

Materials and Methods

Animals

Eight-week-old ICR mice purchased from CLEA (Osaka, Japan) were used in the present study after acclimation for 1 to 2 weeks. The experimental protocols were approved by the Animal Care and Use Committee of Kinki University. Mice were kept under specific pathogen free conditions and housed in polycarbonate cages in a room in which the temperature (23 ± 1 °C), humidity (50 ± 5%) and light cycle (12: 12 h light/dark cycle; lights on at 07.00 hours) were maintained. To avoid the possibility of stressing the animals, noise levels were kept to a minimum both within the room and in the adjacent areas. Mice were allowed food (Certified Rodent Chow CE-2; CLEA, Osaka, Japan) and drinking water *ad libitum*. Certification analysis of each lot of diet was performed by the manufacturer. The same lots of diet were provided to animals from control and compound-exposed groups at the same times, in order to control across groups for possible variation in the content of the diet. Ten-week-old virgin female mice weighing 29–34 g were cohabited for 2 h (07.00 to 09.00 hours) on a 1: 1 basis with males aged 11 weeks or older. Females were checked for the presence of a vaginal plug immediately thereafter, and 08.00 hours was defined as the time of conception if a plug was found (Nagao *et al.*, 2000).

Exposure of Pregnant Dams to DES and Isolation of Placental Tissues

Diethylstilbestrol (DES, (E)-3,4-Bis(4-hydroxyphenyl)-3-hexene) and 17β-estradiol (E_2) were purchased from Sigma Chemical (St. Louis, MO, USA), suspended in corn oil and administered orally on days 4 through to 8 of gestation, a time in the early development of the placenta when ERRβ expression is essential (Tremblay *et al.*, 2001). The dose solution was prepared once per 5 days and analyzed prior to dosing. The DES or E_2 concentration was confirmed to be within ± 10% of the targeted concentration. Mice administered corn oil (5 ml kg^{-1} body weight) were used as controls. Ten mice each were administered DES at 1, 5, 10 or 15 μg kg^{-1} day^{-1}, or E_2 at 50 μg kg^{-1} day^{-1}. Administration occurred at a defined time (12.00 to 12.15 hours). On day 13 of gestation, pregnant mice were euthanized using carbon dioxide anesthesia followed by cervical dislocation, and the uteri from dams in each group were removed quickly to count the numbers of live and dead embryos as well as to measure embryonic and placental weights, and the determination of sex was carried out by observation of the gonadal morphology and position under a dissecting microscope to examine the embryo-sex-dependent differences. Subsequently, the placentas were quickly washed in sterile Tyrode's solution.

Determination of ERα, ERβ, ERRβ or ERRγ mRNA in the Developing Placentas Using RT-PCR

In two placentas with female embryos and two placentas with male embryos of each dam, the junctional zone (decidua basalis and spongiotrophoblasts) and the labyrinth zone were separated using a scalpel under a dissecting microscope (SZX12, Olympus Co., Tokyo, Japan). In the separation of zones, the boundary layer between the spongiotrophoblasts and the labyrinth was excluded from the sample for analysis in order to avoid the mixture of the 'peg' of spongiotrophoblast cells into the labyrinthine part of the placenta. Tissues from each zone in the placentas of the groups exposed to DES at 0, 5 or 10 μg kg^{-1} day^{-1}, and E_2 at 50 μg kg^{-1} day^{-1} were provided for RT-PCR to determine the mRNA levels of ERα, ERβ, ERRβ and ERRγ.

Total RNA was isolated from placental tissues from each zone using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 1 μg of purified total RNA using AMV Reverse Transcriptase XL from the RT-PCR Kit (Takara Bio Inc., Shiga, Japan) with a random primer at 42 °C for 60 min. PCR amplification of ERα, ERβ, ERRβ or ERRγ was carried out on equal quantities of cDNA product (20 μl) using a TaKaRa PCR Thermal Cycler Dice mini (Takara Bio Inc., Shiga, Japan). The sequences of ERα, ERβ, ERRβ and ERRγ primers and conditions of PCR amplification of cDNA are shown in Table 1. PCR products were run on a 1.5% Agarose S gel (Nippon Gene Co., Ltd., Tokyo, Japan) and visualized by ethidium bromide (Nippon Gene Co., Ltd., Tokyo, Japan) staining under ultraviolet (UV) light. Gene expression levels were normalized by the expression levels of β-actin as an internal control.

Table 1. Sequences of PCR primers

Primer name		Sequences	Products (bps)	Annealing temperature
β-actin	Forward	tgttaccaactgggacgaca	392	58 °C
	Reverse	tctcagctgtggtggtgaag		
ERα	Forward	atgaaaggcggcatacggaaag	94	60 °C
	Reverse	cacccatttcatttcggccttc		
ERβ	Forward	ccagactgcaagcccaaatgt	81	60 °C
	Reverse	agaagcgatgattggcagtgg		
ERRβ	Forward	cgctcgctcccatacgat	78	60 °C
	Reverse	ccctaccaggcgagagtgttc		
ERRγ	Forward	actccatgcccaagagactg	137	60 °C
	Reverse	ggctgggcagctgtactcta		

Observation of the Placental Morphology

Three to four placentas of each dam in the groups exposed to DES at 0, 1, 5 or 10 μg kg^{-1} day^{-1}, and E_2 at 50 μg kg^{-1} day^{-1} were fixed for 24 h in 0.1 M phosphate-buffered 10% formalin embedded in paraffin, and sectioned for staining with hematoxylin and eosin. The thicknesses of the whole placenta and labyrinth zone close to the central portion were measured to calculate the proportions of labyrinth zone per whole placenta (Fig. 1). Subsequently, the number of TGCs in the trophoblast layer between the spongiotrophoblast layer and decidua basalis was counted using a calibrated ocular grid in all visual fields with × 200 objective magnification. Three separate fields were randomly selected in each placenta and the number of TGCs was counted according to the methods previously reported (Suzuki *et al.*, 1997).

For ultrastructural observation, one to two placentas in each dam in the groups exposed to DES at 0, 1, 5 or 10 μg kg^{-1} day^{-1}, and E_2 at 50 μg kg^{-1} day^{-1} were selected at random. The placentas from the uteri were fixed in 0.1 M phosphate-buffered 2% paraformaldehyde and 1.25% glutaraldehyde (pH 7.4). After fixation, placentas were rinsed three times in phosphate buffer, post-fixed for 2 h at 4 °C in 0.1 M phosphate-buffered 2% osmium tetroxide and dehydrated in alcohol. Placentas were embedded in epoxy resin. Semithin sections (about 1 μm) were stained with toluidine blue, and histopathological morphology was observed. Ultrathin sections stained with uranyl acetate and lead citrate were prepared from a representative area of the basal zone of the placenta and TGCs were observed in the trophoblast layer between the spongiotrophoblast layer and decidua with an electron microscope (HT7700, Hitachi, Japan).

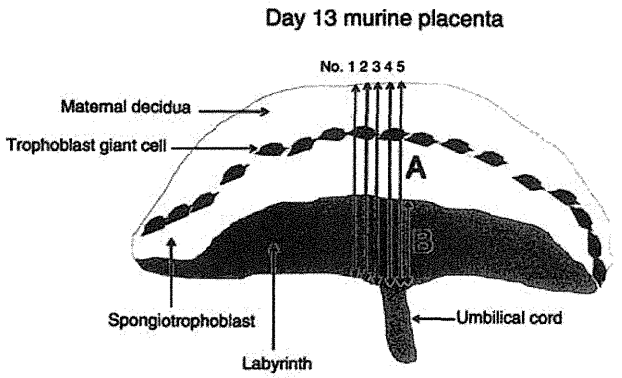

Figure 1. Diagrammatic transverse section of placenta showing the measurement of thickness. The measurement was performed with three sections close to the central portion from each placenta, and five areas (No. 1–5) close to the highest portion in each section. Fifteen values of A, B or B/A in each placenta were obtained, and the mean value of A, B or B/A in each placenta was determined. Two to three placentas per dam were used. (A) thickness of whole placenta, (B) thickness of labyrinth zone.

Statistical Analysis

Data are given as the mean ± standard deviation (SD). Comparisons between the treated groups and controls were performed using $P \leq 0.05$ and $P \leq 0.01$ as levels of significance. Mean fetal weight and placental weight, and the number of TGCs were compared using Student's *t*-test. The proportions of resorbed embryos and labyrinth zone/whole placenta were compared using the Mann–Whitney *U*-test as described by Siegel (1956) to determine the significance of differences. As for RT-PCR, statistical analysis was performed using SPSS (ANOVA followed by post-hoc analysis [LSD]) when the test for homogeneity of variance was fulfilled and using Games-Howell post-hoc analysis when the homogeneity of variance was not attained.

Results

Embryonic Mortality, and the Weight of Embryos and Placentas

No adverse effects on the general conditions: decreased body weight gain, death, moribund, loss of spontaneous activity, crouching, tremor, piloerection, loose stool and so on, in maternal animals were observed in any DES- or E_2-exposed groups. The developmental effects of DES and E_2 on the mortality of embryos, and embryonic and placental weights by sex, when evaluated on days 13 of gestation, are presented in Table 2. After oral exposure to DES on days 4 through to 8 of gestation, the embryonic mortality in the groups treated with DES at 10 or 15 μg kg^{-1} day^{-1} was significantly increased compared with that of the controls. Most of the dead embryos in the group exposed to DES at 10 μg kg^{-1} day^{-1} were resorbed at the early to middle developmental stage. Dead embryos in the group exposed to DES at 15 μg kg^{-1} day^{-1} almost all involved early resorptions, or implantation sites, suggesting death immediately after early administration. Embryonic mortality in the group exposed to

Table 2. Embryonic mortality, placental weight and embryonic weight in dams exposed to diethylstilbestrol (DES) or 17β-estradiol (E_2) on days 4 through to 8 of gestation

Dose ($\mu g\ kg^{-1}\ day^{-1}$)	No. of dams	Embryonic mortality (%)	No. of dams with dead embryos	Placental weight (g, A)		Embryonic weight (g, B)		B/A	
				Male	Female	Male	Female	Male	Female
DES 0	10	2.8 ± 4.1[a] (4/117)[b]	2 (0)[c]	0.096 ± 0.013 (75)[d]	0.091 ± 0.014 (68)[d]	0.183 ± 0.034 (75)[e]	0.178 ± 0.031 (68)[e]	1.919 ± 0.339	1.937 ± 0.333
1	10	5.4 ± 6.3 (8/150)	1 (0)	0.087 ± 0.009 (76)[f]	0.084 ± 0.005 (64)	0.220 ± 0.036 (78)	0.208 ± 0.035 (64)	2.290 ± 0.491	2.276 ± 0.535
5	10	5.5 ± 5.2 (8/145)	2 (0)	0.094 ± 0.010 (64)	0.088 ± 0.012 (73)	0.173 ± 0.014 (64)	0.167 ± 0.014 (73)	1.839 ± 0.307	1.894 ± 0.281
10	10	68.5 ± 44.7** (104/151)	9 (2)	0.100 ± 0.006 (27)	0.095 ± 0.007 (20)	0.177 ± 0.013 (27)	0.169 ± 0.016 (20)	1.773 ± 0.115	1.781 ± 0.203
15	10	88.2 ± 26.5** (121/137)	10 (6)	0.105 ± 0.008 (9)	0.096 ± 0.008 (7)	0.163 ± 0.018 (9)	0.165 ± 0.007 (7)	1.550 ± 0.118**	1.628 ± 0.136*
E_2 50	10	4.91 ± 2.86 (7/152)	1 (0)	0.093 ± 0.013 (68)	0.090 ± 0.009 (77)	0.193 ± 0.018 (68)	0.188 ± 0.017 (77)	2.008 ± 0.303	1.998 ± 0.258

[a]Mean ± SD
[b]No. of dead embyos/no. of implants
[c]No. of dams with no survivors.
[d]No. of placentas with male or female embryos.
[e]No. of male or female embryos.
[f]Two placentas were not used for further analysis because they were fused *in utero*.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

DES at 1 or 5 μg kg^{-1} day^{-1} was comparable to that of the controls. No significant increase in the mortality was observed in the group exposed to E_2 at 50 μg kg^{-1} day^{-1}.

There were no significant differences between the DES-treated groups and the controls in terms of weights of placentas and embryos in both sexes, whereas the placental weight showed a tendency to increase and the embryonic weight showed a tendency to decrease in the group exposed to DES at 10 or 15 μg kg^{-1} day^{-1} in comparison with those in the controls. The placental or embryonic weight in both sexes of the E_2-treated group was comparable to that in the control.

ER or ERR mRNA Expression in the Placentas

Expression of ERα and ERβ mRNA, and ERRβ and ERRγ mRNA in the placentas of dams exposed to DES or E_2 is shown in Fig. 2.

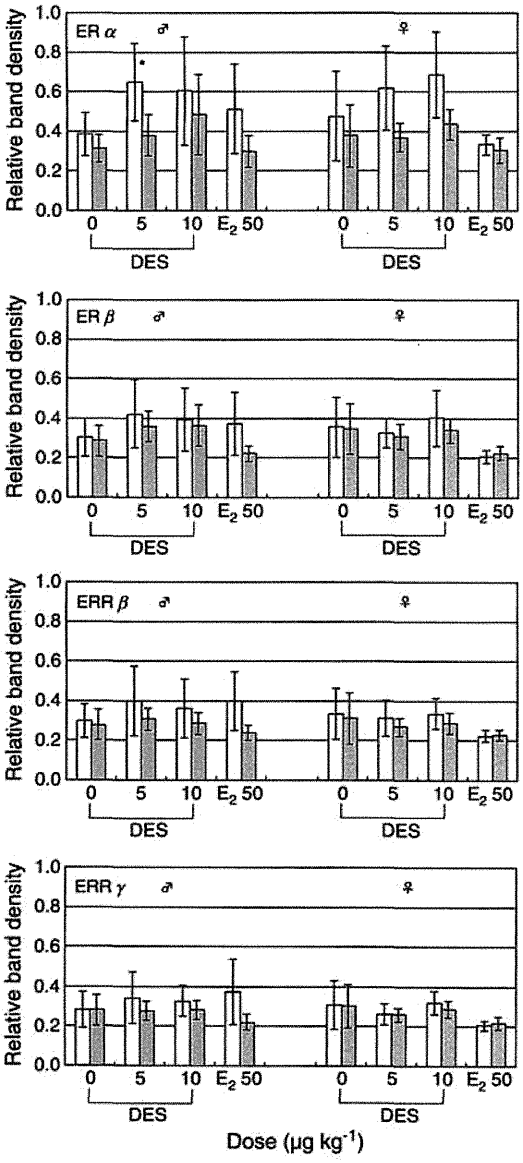

Figure 2. The expression of ERα, ERβ, ERRβ and ERRγ mRNA in the junctional zone (□) and labyrinth zone (■) of placentas with male or female embryos of dams exposed to diethylstilbestrol (DES) or 17β-estradiol (E_2) on days 4 through to 8 of gestation. *Significantly different from the control, $P < 0.05$.

Expression of ERα mRNA in the junctional zone (decidua basalis and spongiotrophoblasts) of placentas with male embryos in the group exposed to DES at 5 μg kg^{-1} day^{-1} was significantly higher than that in the control ($P = 0.003$). No significant differences in any ERα, ERβ, ERRβ and ERRγ mRNA expression were observed between the group exposed to DES at 10 μg kg^{-1} day^{-1} or the group exposed to E_2 at 50 μg kg^{-1} day^{-1} and the controls in both decidua basalis with spongiotrophoblasts and the labyrinth zone of the placentas with male embryos. In the placentas with female embryos, there were no significant differences in the mRNA expression of ERα, ERβ, ERRβ and ERRγ between the groups exposed to DES or E_2 and the controls.

Morphology of Placenta

Light microscopy of the placenta clearly showed that the group exposed to DES at 10 μg kg^{-1} day^{-1} lacked the developing labyrinth (Fig. 3). The decidua was much less affected than the labyrinth in the DES-exposed group, but decidual cells were smaller than in the controls. Ultrastructural changes in the TGCs of the placentas sampled on day 13 of gestation are shown in Fig. 4. There were rich rough-surfaced endoplasmic reticulum and Golgi apparatus in the TGCs of the group exposed to DES at 1 or 5 μg kg^{-1} day^{-1} or E_2 at 50 μg kg^{-1} day^{-1}, and in the control group (Fig. 4A). In the group exposed to DES at 10 μg kg^{-1} day^{-1}, poor rough-surfaced endoplasmic reticulum or its atrophy was found (Fig. 4B).

The decidua basalis, spongiotrophoblasts and labyrinth zone were easily discriminated for both control and DES-treated groups. The developmental parameters of placenta are shown in Table 3. The thickness of the whole placenta and that of the labyrinth zone in the group treated with DES at 10 μg kg^{-1} day^{-1} were significantly decreased compared with those of the controls. Those in the group exposed to DES at 1 or 5 μg kg^{-1} day^{-1} or E_2 at 50 μg kg^{-1} day^{-1} were comparable to those of the controls. No significant differences were found in the proportions of the labyrinth zone per whole placenta between the DES- or E_2-treated groups and the controls. Numbers of TGCs per unit area of decidua basalis and spongiotrophoblasts are also shown in Table 3. The number of TGCs in the group exposed to DES at 10 μg kg^{-1} day^{-1} was significantly increased compared with that in the controls. There were no significant differences in the number between the group exposed to DES at 1 or 5 μg kg^{-1} day^{-1} or E_2 at 50 μg kg^{-1} day^{-1} and the controls.

Discussion

It is well known that estrogen administration elicits hemorrhage around gestational sacs and decreases placental blood flow in the mouse and rabbit (Abdul-Karim and Bruce, 1972; Mahendroo *et al.*, 1997; Scott and Adejokum, 1980). In our previous study, we demonstrated that decidual hypoplasia and subsequent placental hemorrhage cause fetal death as a result of the oral administration of DES during the early stage of pregnancy in mice (Nagao and Yoshimura, 2009).

The present study clearly showed that placental development was affected after oral exposure to DES, resulting in prenatal lethality. It has been reported that DES acts in rats to depress the preterm levels of steroid hormones, which leads to a failure of uterine contraction accompanied by placental detachment and fetal death (Clevenger *et al.*, 1991; Zimmerman *et al.*, 1991). Bartholomeusz *et al.* (1999) demonstrated a decrease in embryo

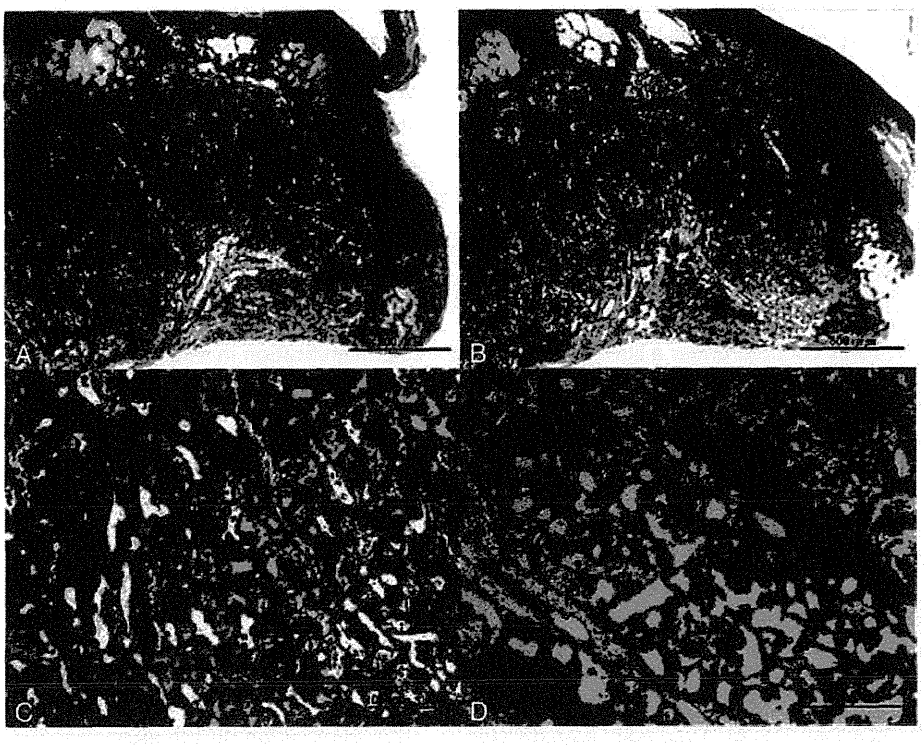

Figure 3. Light microscopic profiles of transverse sections of the control placenta (A, C) of a dam on day 13 of gestation showing a rich villous network and of the placenta (B, D) of a dam exposed to diethylstilbestrol (DES) at 10 μg $^{-1}$kg day^{-1} on days 4 through to 8 of gestation showing impaired villous network. Upper panels (A, B) show the peripheral part of placentas and the labyrinth zone (L) was magnified (C, D). Toluidine blue staining.

survival and disturbance of fetal and placental growth after elevation of maternal estradiol blood concentration in rats.

Embryonic Mortality and Placental Growth

The incidences of embryonic mortality after treatment with DES at 0, 1, 5, 10 and 15 μg kg^{-1} day^{-1} were 2.8%, 5.4%, 5.5%, 68.5% and 88.2%, respectively, suggesting that the no observed adverse effect dose level (NOAEL) for embryonic lethality was 5 μg kg^{-1} day^{-1} upon oral exposure on days 4 through to 8 of gestation and evaluation on day 13 in ICR mice. In the 1940s, DES was used to prevent adverse pregnancy outcomes in women with a history of miscarriage. Evaluating the DES applied dose as 5–125 mg day^{-1} (Coscrove *et al.*, 1977; Oradell, 1961), a 150-pound (assuming an average body weight of 68 kg) pregnant woman could intake 74–1800 μg kg^{-1} day^{-1}, and the dose is 15–360 times higher than the NOAEL for embryonic lethality evaluated in the present study. In a previous study, it was reported that the embryonic mortality after oral exposure to DES at 10 μg kg^{-1} day^{-1} on days 4 through to 8 of gestation was approximately 25% when evaluated on day 9 of gestation in mice (Nagao and Yoshimura, 2009). Thus, it is suggested that many embryos of dams exposed to DES at this dose on days 4 through to 8 proceeded to death during days 9 to 13 of gestation.

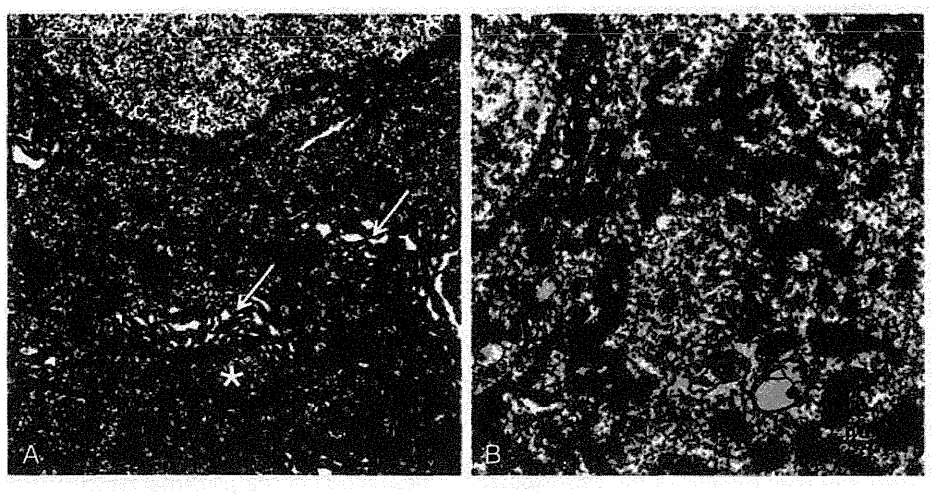

Figure 4. Electron micrographs of trophoblast giant cells in the control placenta (A) of a dam on day 13 of gestation showing rich rough-surfaced endoplasmic reticulum (*) and Golgi apparatus (↓), and in the placenta (B) of a dam exposed to diethylstilbestrol (DES) at 10 μg kg^{-1} day^{-1} on days 4 through to 8 of gestation showing depletion of rough-surfaced endoplasmic reticulum.

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Table 3. Developmental effects of diethylstilbestrol (DES) or 17β-estradiol (E_2) on labyrinth and trophoblast giant cells (TGCs)

Dose (μg kg $^{-1}$ day^{-1})	Thickness of whole placenta (A)	Thickness of labyrinth zone (B)	B/A × 100	Number of TGCs[b]
DES 0	1382.9 ± 156.4[a]	708.3 ± 142.0[a]	51.3 ± 8.4	14.8 ± 4.4
1	1395.0 ± 147.1	727.3 ± 120.9	52.7 ± 7.8	13.6 ± 3.7
5	1405.6 ± 155.0	765.3 ± 173.9	54.2 ± 9.8	12.3 ± 4.4
10	1284.6 ± 156.2**	634.0 ± 126.5**	49.4 ± 8.1	19.6 ± 3.6*
E_2 50	1409.1 ± 94.7	688.6 ± 80.3	49.0 ± 6.0	16.3 ± 3.9

[a]Mean ± SD, μm.
[b]The number of TGCs was counted under a light microscope at × 200 magnification in three areas (decidua basalis and spongiotrophoblasts) randomly selected from three to four placentas of each dam.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

Oral exposure to DES at 10 or 15 μg kg^{-1} day^{-1} resulted in a high frequency of resorptions of embryos. Interestingly, a significant increase in the resorption did not occur when E_2 at a fairly high dose of 50 μg kg^{-1} day^{-1} was applied (4.9%). Orally administered DES is rapidly absorbed, distributed and cleared in maternal organs, and its major site of accumulation is the liver of mice (Shah and McLachlan, 1976). Maternally administered DES crosses the placenta and is rapidly distributed to the fetus in rats, mice, hamsters and monkeys (Fischer *et al.*, 1976; Hill *et al.*, 1980; Maydl and Metzler, 1984; McLachlan, 1977); the fetal level is 2–3 times higher than in the maternal blood (Miller *et al.*, 1982). Estradiol given orally in its native crystalline state is poorly absorbed via the gastro-intestinal tract because of extensive first-pass metabolism (Lobo, 1987). Estrogen given orally in mice does not produce continuously elevated levels of estrogen and is rapidly metabolized to control levels (Gordon *et al.*, 1986).

The biological activity of estrogens is influenced by the degree to which they bind to serum proteins. DES binds poorly to sex hormone binding globulin (SHBG), and DES showed enhanced access in serum (serum modified access, SMA = 6.2). SMA was calculated by dividing the relative binding affinity (RBA, relative to estradiol) measured in 100% serum by the RBA measured in serum-free medium. An SMA > 1 indicated that the xenoestrogen had greater access to estrogen receptors than estradiol from serum. Additional calculations through Ki (inhibition constant) indicated that this corresponded to an effective free fraction of 26.9% for DES in serum (Nagel *et al.*, 1998). Therefore, at equivalent concentrations of estradiol and DES, the concentration of DES at the target cell will be significantly greater than that of estradiol. Arnold *et al.* (1996) demonstrated that DES induced greater β-galactosidase activity than estradiol in the presence of albumin or SHBG, suggesting that the availability of DES is greater than estradiol in the presence of albumin or SHBG (the IC50 for SHBG was 0.035 mg ml^{-1} for estradiol and 0.15 mg ml^{-1} for DES).

As for the placental and embryonic weight on day 13 of gestation, the weight of male embryos on day 13 of gestation was greater than that of females in control and DES-treated groups. These results are compatible with those in CD-1 mice reported by Ishikawa *et al.* (2006). Although there were no significant differences in the placental and fetal weights of the DES-exposed groups compared with those in the controls, the trends in fetal and placental weights were opposite. The ratios of fetal: placental weights were increased in a dose-dependent manner, and significant increases were detected between the 15 μg kg^{-1} day^{-1} group and the controls in both sexes, suggesting that oral DES dosing affects the development of embryos (see Table 2).

Effects of DES on the Expression of ER and ERR mRNA

Little information on the expression profile of ER mRNA including ERR mRNA in developing rodent placentas is available. Immunocytochemistry revealed that ER was localized within nuclei of cells of the mesometrial decidua basalis but not in trophoblastic cells (day 10 of gestation) or in cells of the junctional zone or labyrinth zone in the rat (Ogle and George, 1995). In murine placentas, ERβ mRNA expression was demonstrated in decidual cells (Kurita *et al.*, 2001). RT-PCR analysis revealed that DES exposure caused an increase in ERα mRNA in the cells of the mesometrial decidua basalis with spongiotrophoblasts but not in cells of the labyrinth zone (see Fig. 2). As for ERβ mRNA, there was no change in the expression pattern in the placenta exposed to DES.

The present findings on the disturbance of placental development by DES and earlier studies (Luo *et al.*, 1997; Tremblay *et al.*, 2001) led us to hypothesize that ERRβ has an important role in early placentation. To test this hypothesis, we investigated the expression of ERRβ mRNA by RT-PCR in the placentas from dams on day 13 of gestation. The present study showed that exposure to DES at 10 μg kg^{-1} day^{-1} did not change the expression profile of ERRβ mRNA in the junctional and labyrinth zone (see Fig. 2), although DES at this dose induced embryonic mortality at a high frequency. Thus, it is reasonable to suggest that the high rate of embryonic mortality induced by DES is not associated with the ERRβ mRNA expression in mouse placenta.

Morphology of Placenta and Ultrastructural Changes of TGCs

The treatment of mice with DES (12.5 μg kg^{-1} day^{-1}) during mid-gestation (days 9 through to 12 of gestation) induces placental changes that include thinning of the labyrinth zone and accumulation of TGCs (Scott and Adejokun, 1980). In addition, the treatment of rats with estradiol benzoate from days 12 through to 19 of gestation induces trophoblast degeneration and destruction of the placental labyrinth (Matsuura *et al.*, 2004). In the present study, light microscopy of the placenta exposed to DES from days 4 through to 8 of gestation showed the impaired development of the labyrinth where most gaseous and nutrient

exchange occurs (see Fig. 3). Thus, it is suggested that disturbance of the villous network resulting from the reduced villigenesis might be associated with the decreases in the physiological function of the placenta.

TGCs are the first terminally differentiated cell type to form during embryogenesis in rodents and are of vital importance for embryo implantation and the promotion of maternal adaptations to pregnancy. TGCs examined in the present study line the implantation site and are in direct contact with decidual and immune cells in the uterus (Simmons *et al.*, 2007), and have several functions to facilitate implantation and initial maternal vascular connections, as well as to regulate decidual cell differentiation and maternal physiology (Hu and Cross, 2010). TGC differentiation can be induced by DES (Tremblay *et al.*, 2001) and retinoic acid (Yan *et al.*, 2001). When injected into pregnant mice, both of these compounds cause an overabundance of giant cells at the expense of other trophoblast subtypes such as stem cells and spongiotrophoblasts, suggesting the differentiation-promoting activity of retinoic acid and DES. This differentiation-promoting activity of retinoic acid and DES is also observed in trophoblast stem cells *in vitro* (Tremblay *et al.*, 2001; Yan *et al.*, 2001). In addition, in the present study, the number of parietal TGCs of the placenta exposed to DES was significantly increased compared with that in the controls, whereas E_2 treatment did not increase the number of TGCs. A few transcription factors have been suggested to regulate giant-cell formation, such as two members of the basic helix-loop-helix (bHLH) family, *Mash2* and *Hand1*. The targeted deletion of this gene results in an increase in the number of giant cells at the expense of the spongiotrophoblasts (Guillemot *et al.*, 1994; Tanaka *et al.*, 1997). In a previous study (Nagao and Yoshimura, 2009), effects on mouse developing placentas were evaluated on day 9 of gestation after exposure to DES at 10 $\mu g\ kg^{-1}\ day^{-1}$ on days 4 through to 8, and the number of implantation sites per litter in the DES group (14.8 ± 1.9) was similar to that in the control (14.6 ± 2.3). This suggests that the increase in TGCs in the DES group seen in the present study may be related to the facilitation of implantation.

In the ultrastructural observation of TGCs, poor rough-surfaced endoplasmic reticulum and its atrophy were found in the TGCs of the placentas of mice exposed to DES at 10 $\mu g\ kg^{-1}\ day^{-1}$, suggesting the association with the disruption of synthesis of protein. E_2 at 50 $\mu g\ kg^{-1}\ day^{-1}$ or DES at 1 or 5 $\mu g\ kg^{-1} day^{-1}$ did not induce changes of ultrastructure of TGCs or a high frequency of embryonic mortality.

The placenta is a unique organ; placentation starts at the time of implantation and its functional role terminates at the time of parturition. Recently, a few studies have examined the role of endocrine-disrupting chemicals (EDCs) in fetal growth restriction and pregnancy loss, but evidence suggests that exposure to some EDCs during pregnancy can contribute to incomplete placentation. In laboratory animal studies, early exposure to estrogens has been shown to induce trophoblast degeneration (both apoptosis and placental labyrinth destruction) in pregnant rats treated with a physiological dose of estradiol benzoate during days 12 through to 19 of gestation (Matsuura *et al.*, 2004). On day 20 of gestation, the exposed embryos had reduced weight compared with the control ones, indicating fetal growth restriction associated with trophoblast degeneration. Poor placentation, miscarriage and increased neonatal mortality were also observed in mice exposed during early gestation to bisphenol A (Tachibana *et al.*, 2007). These *in vivo* studies suggest that early estrogen or xenobiotic exposure could limit trophoblast invasion of the endometrium, placing the fetus at risk of intrauterine growth restriction or prenatal and neonatal mortality. However, more studies are needed to understand the developmental effects of inappropriate hormones or xenobiotic exposure on first-trimester placentation.

In conclusion, oral exposure to DES during early to middle gestation affects the rough-surfaced endoplasmic reticulum of TGCs and the developing labyrinth, and it is suggested that these developmental changes may be related to decreased protein synthesis or disruption of nutrition and oxygen exchange between mother and embryo, resulting in a high frequency of embryonic mortality.

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Maternal bisphenol A oral dosing relates to the acceleration of neurogenesis in the developing neocortex of mouse fetuses

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ABSTRACT

Bisphenol A (BPA), an endocrine-disruptor, is widely used in the production of plastics and resins. Human perinatal exposure to this chemical has been proposed to be a potential risk to public health. Animal studies indicate that postnatal exposure to BPA may affect neocortex development in embryos by accelerated neurogenesis and causing neuronal migration defects. The detailed phenotypes and pathogenetic mechanisms, especially with regard to the proliferation and differentiation of neural stem/progenitor cells, however, have not been clarified. C57BL/6J pregnant mice were orally administered BPA at 200 μg/kg from embryonic day (E) 8.5 to 13.5, and the fetuses were observed histologically at E14.5. To clarify the histological changes, especially in terms of neurogenesis, proliferation and cell cycle, we performed histological analysis using specific markers of neurons/neural stem cells and cell cycle-specific labeling experiments using thymidine-analog substances. Cortical plate was hyperplastic and the number of neural stem/progenitor cells was decreased after the exposure to BPA. In particular, the maternal BPA oral dosing related to the effects on intermediate progenitor cells (IPCs, neural progenitor cells) in the subventricular zone (SVZ) of dorsal telencephalon. Exposure to BPA associated the promotion of the cell cycle exit in radial glial cells (RGCs, neural stem cells) and IPCs, and decreased the proliferation resulting from the prolong cell cycle length of IPCs in the SVZ. Our data show that maternal oral exposure to BPA related to the disruption of the cell cycle in IPCs and the effects of neurogenesis in the developing neocortex.

1. Introduction

Endocrine-disrupting industrial chemicals are released into the environment and interfere with normal endocrine function. Bisphenol A (BPA; 2,2-bis(4-hydroxy-phenyl) propane) is known to be one of the endocrine-disrupting chemicals because of its weak estrogenic, androgenic and thyroid hormone-like activity (Hiroi et al., 2006; Krishnan et al., 1993; Takayanagi et al., 2006; Xu et al., 2005). BPA is used in polycarbonate plastics, epoxy resins, and dental resin-based composites (Howe and Borodinsky, 1998; Pulgar et al., 2000; Sasaki et al., 2005). BPA has been detected in the serum of pregnant women (1–2 ng/ml), fetus serum (0.2–9.2 ng/ml), amniotic fluid (8.3–8.7 ng/ml), placental tissue (1.0–104.9 ng/ml), and breast milk (1.1 ng/ml) (Ikezuki et al., 2002; Schonfelder et al., 2002; Ye et al., 2005), suggesting that the human fetus is exposed to this compound during pre- and post-natal development. Several studies reported some adverse effects of high- and low-dose BPA on various organs during the prenatal period (Golub et al., 2010; Kundakovic and Champagne, 2011). During fetal development, there is a sensitive period during which environmental exposure may cause persistent damage to the developing brain. Maternal exposure to low-dose BPA (20 μg/kg/day, intraperitoneally) has been shown to affect cortical development in embryos by accelerated neurogenesis and neuronal migration during the mid-gestational period in mice (Nakamura et al., 2006). In addition, BPA exposure results in abnormal neuronal positioning and aberrant neuronal network formation between the thalamus and the cortex in the mature brain exposed prenatally (Nakamura et al., 2007). However, no findings have been added to these reports about the morphological changes of the neocortex induced by low-dose BPA exposure.

During neocortical development, neural stem/progenitor cells sequentially pass through phases of expansion, neurogenesis and gliogenesis. Radial glial cells (RGCs) in the ventricular zone (VZ), neural stem cells, expand their population by symmetric

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division that produces two RGCs and simultaneously self-renew and generate more differentiated cells through asymmetric cell division (Gotz and Huttner, 2005). The daughter cells of ventricular RGCs are often neuronal progenitor cells and can migrate superficially into the subventricular zone (SVZ) to divide (Haubensak et al., 2004; Noctor et al., 2004). RGC divisions are asymmetric and associated with self-renewal, while the daughter progenitor cells (intermediate progenitor cells: IPCs) usually undergo one terminal symmetric division that produces two neurons and depletes the progenitor cells (Noctor et al., 2004). The majority of layer II/III neuron-producing cell divisions are by IPCs during the late neurogenesis stages (Kowalczyk et al., 2009). The number of symmetric IPC divisions could also affect neuron number and be a determinant of neocortical size. A human genetic study pinpointed the disease-causing gene in a family exhibiting congenital microcephaly to the homozygous silencing of Tbr2 (Baala et al., 2007), a transcriptional factor shown in rodent studies to be a selective marker for IPCs (Englund et al., 2005). These reports suggest the overall importance of IPCs of SVZ in the control of neocortical size.

The purposes of this study are to clarify the mechanisms underlying the abnormal proliferation and differentiation of neural stem cells in fetuses exposed to BPA. Observation of the BPA-treated fetuses revealed the accelerated neurogenesis and a reduced number of neural stem/progenitor cells, especially IPCs, in the dorsal telencephalon. Our data suggested that these aberrations of developing neocortex result in disruption of the cell cycle in IPCs.

2. Materials and methods

2.1. Animals and housing

Eight-week-old male and female C57BL/6J mice were purchased from Japan SLC Inc. to be used as breeder animals in this study. These mice were quarantined and habituated for 2 weeks. These animals were kept under SPF conditions and a constant light–dark cycle (dark period from 7:00 pm to 7:00 am) at 24 ± 1 °C and 55 ± 5% relative humidity. To avoid the possibility of stressing the animals, noise levels were kept to a minimum both within the room and in the adjacent areas. Diet food (Certified Rodent Chow CE-2, CLEA Japan) and drinking water were available as libitum. Certification analysis of each lot of diet was performed by the manufacturer. The same lots of diet were provided to animals from control and BPA-treated groups and at the same times, in order to control across groups for possible variation in the content of the diet. Water was available via glass bottles with Teflon seals during the exposure period. Pregnant females were housed individually throughout the study in polypropylene plastic tubs with stainless steel lids and corncob bedding. Mice in all experiments were humanely treated according to the guidelines of the Animal Research Committee of Kinki University. Ten-week-old mice were allowed to copulate overnight at a 2:1 or 1:1 female to male ratio. Females were checked at 12 h intervals for the presence of vaginal plugs, indicating copulation, and were separated from the male if a plug was present. The presence of a plug represented embryonic day (E) 0.

2.2. Test substance and treatment regimen

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane 4,4′-isopropylidenediphenol, CAS no. 80-05-7, Sigma Aldrich) was suspended in corn oil and administered by oral gavage from E8.5 to E13.5. The dose solution was prepared once per 5 days and analyzed prior to dosing. The BPA concentration was confirmed to be within ±10% of targeted concentration. Administration occurred at a defined time (12:00 pm). In a preliminary study, three dams per group were exposed orally to BPA at 20 or 200 μg/kg from E8.5 through E13.5, and killed on E14.5 to collect the fetuses. Nine female fetuses in each group were examined histologically in terms of the developing brain (Sup. Table 1). No histological abnormality, such as hyperplasia of cortical plate and the acceleration of cell cycle exit (Sup. Fig. 1, Tables 1 and 2), were detected in the fetuses exposed to BPA at 20 μg/kg. In the fetuses exposed to BPA at 200 μg/kg, hyperplasia of cortical plate and the promotion of neurogenesis were identified. On the basis of these results, BPA dose used in the present study was chosen as 200 μg/kg/day, which revealed clear effects on the development of neocortex. Control animals received an equal volume of corn oil. At this dose of BPA (200 μg/kg/day) we calculate intake to be approximately 3 μg of BPA daily; body weight of mice used was approximately 30–40 g during the administration period. In the previous study, when rat dam was orally administered BPA at 6 μg/kg/day, 4–5 μg/L BPA was detected in embryonic serum (Yoshida et al., 2004). In addition, prenatal exposure to BPA at 200 μg/kg/day BPA induced changes of embryonic body weight and total number of embryos born per litter (Cagen et al., 1999). These data indicate that the embryos were exposed to BPA in utero in the experimental conditions of the present study.

2.3. Body weight measurement and tissue preparation

Pregnant mice were humanely killed and underwent cesarean section on E14.5, and the fetuses were observed histologically. Fetuses were sampled out of the ostium of the uterus, because there were shown to be no differences in the postnatal growth of the reproductive and endocrine systems, sexual maturation and estrous cycle, or behavior depending on the embryo position in utero (Nagao et al., 2004). For body weight measurement, the body weight before fixation was measured for E14.5 mouse fetuses. Fourteen males and 17 female fetuses from 6 litters in the BPA-treated group and 9 males and 12 females from 6 litters in the control group were used for head size and body weight measurement (Sup. Table 1). Fetuses were fixed for 3 h in periodate lysin paraformaldehyde (PLP) at 4 °C, and washed in phosphate-buffered saline (PBS). For paraffin sections, fetuses were embedded in paraffin and sectioned at 5 μm for histological and immunohistochemical observation. For frozen sections, fetuses were embedded in 30% sucrose/PBS for cryoprotection, and sectioned at 10 μm for immunohistochemical observation. Nine female fetuses from 3 litters in both BPA-treated and control groups were sampled on E14.5 for HE staining and immunostaining (Sup. Table 1). Fetal body weight and brain measurement data were analyzed employing Student's *t*-test.

2.4. Immunohistochemistry

The following antibodies were used: mouse monoclonal anti-neural class III β-tubulin (Tuj1, 1:500; Covance); pan-neuronal marker, rabbit monoclonal anti-Ki67 (1:200; Lab Vision, SP6); a proliferative cell marker, rat monoclonal anti-BrdU (CldU) (1:50; Oxford Biotechnology, BU1/75), mouse monoclonal anti-BrdU (IdU) (1:50; Becton Dickinson, B44), rabbit polyclonal anti-Tbr2 (1:200, Abcam); transcriptional factor and IPC marker, rabbit polyclonal anti-Pax6 (1:200, Covance); transcriptional factor and RGC marker, mouse monoclonal anti-Nestin (1:200, BD Pharmingen); marker of radial fiber in RGC. Secondary antibodies were conjugated with Alexa 568 and 488 (1:200; Invitrogen). The nuclei were counterstained with DAPI in mounting medium (Vector Labs). Immunohistochemistry was performed as described previously (Komada et al., 2008), and standard immunostaining procedures were used in E14.5 fetuses. Nine female fetuses from 3 litters in the BPA-treated group and 9 female fetuses from 3 litters in the control group were sampled on E14.5 for immunostaining (Sup. Table 1).

2.5. CldU and IdU incorporation

For in vivo labeling of S-phase cells (thymidine analog incorporation), one injection of CldU (105478, MP Biomedicals Inc.) and IdU (17125, Sigma) was made 24 h and 1 h, respectively, prior to cesarean sectioning on E14.5. Fetuses were allowed to develop to E14.5 and then sacrificed and processed for CldU and IdU immunohistochemistry. The quantification of positive cells and their distribution within the cortical layer were analyzed according to the methods of Komada et al. (2008), with 2 anatomically matched sections from each fetus (9 BPA-treated and 9 control female embryos).

2.6. Cell cycle kinetics (analysis of cell cycle length and cell cycle exit)

For estimation of cell cycle length, we counted the number of IPCs labeled by 1 h pulse of IdU in Tbr2-positive cells. The population of IdU/Tbr2 double-positive cells among all Tbr2-positive cells enables estimation of the cell cycle length of IPCs. A smaller population of IdU-positive cells among Tbr2-positive cells indicates a greater cell cycle length (Chenn and Walsh, 2002).

Cell cycle exit was estimated from the ratio of CldU-positive cells/Ki67, Pax6, or Tbr2-negative (postmitotic and/or differentiation) cells to all CldU-positive cells at E14.5 in the dorsal telencephalon (Ki67) or upper SVZ (Pax6 and Tbr2) (Chenn and Walsh, 2002). One injection of CldU was made 24 h prior (at E13.5) to sampling (at E14.5).

2.7. Quantification of cell number for cell cycle exit, proliferation, RGC, IPC, and cell cycle length

For quantification of data obtained using immunofluorescent staining, the counterpart areas in the dorsal telencephalic region of brain of the control and BPA-treated fetuses were selected, and the total number of cells (DAPI-stained) and cells stained with each antibody were manually counted in two 100 μm-wide sampling box (indicated by open boxes in Figs. 2–6) with 2 anatomically matched sections from each fetus (9 BPA-treated and 9 control female embryos) using Adobe photoshop CS4 (Adobe). We performed the counting and quantitative procedures by the method reported previously (Komada et al., 2008).

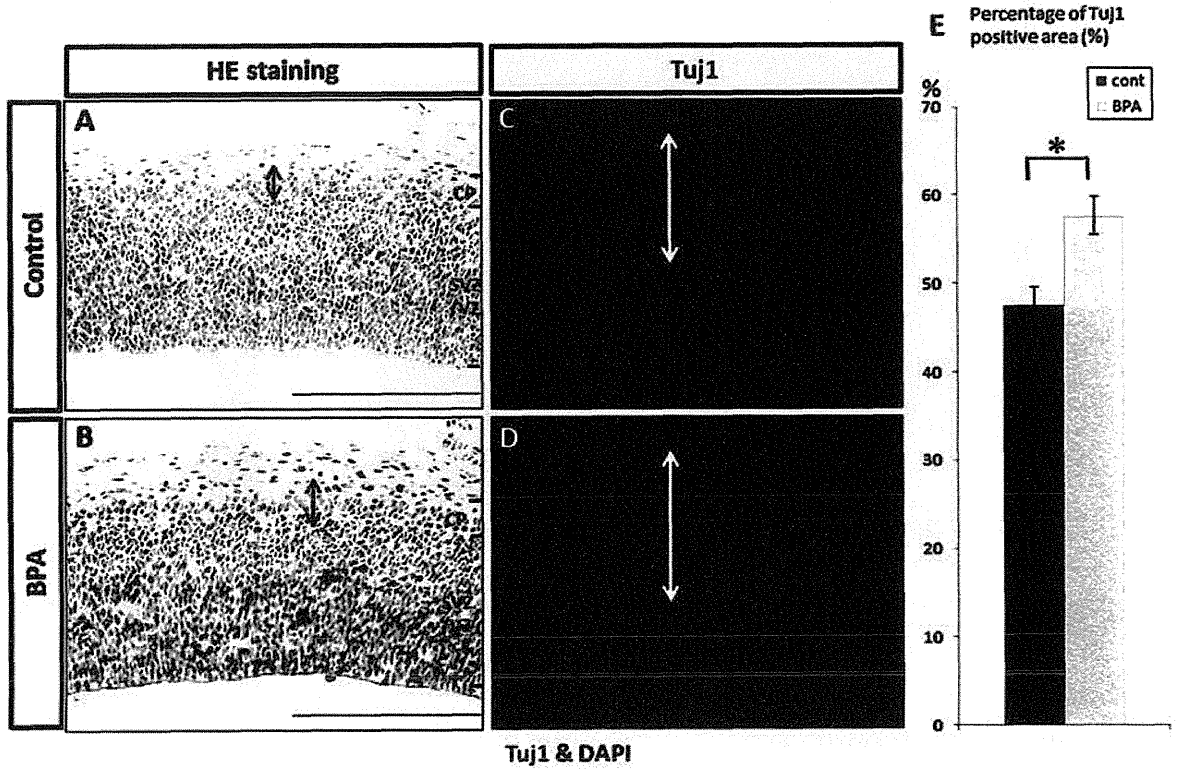

Fig. 1. Maternal BPA oral dosing induces hyperplasia of cortical plate and promotes neurogenesis. Parasagittal sections of the telencephalons of the control fetuses (A) and BPA-treated fetuses (B) at E14.5 were stained with HE. These panels illustrated that the size of the cortical plate was reduced in BPA-treated fetuses (black arrow). Parasagittal sections of E14.5 dorsal telencephalon were immunostained with anti-neuron-specific class III β-tubulin (Tuj1) antibody (C, D) and expressing regions were measured on the sections (white arrow). (E) Tuj1-stained regions were significantly increased in the dorsal telencephalon of BPA-treated fetuses (57.6 ± 2.11%, $n = 9$, $^*P < 0.05$) compared with those of control fetuses (47.5 ± 2.09%, $n = 9$). Scale bar: 200 μm.

3. Results

3.1. Hyperplasia of cortical plate in BPA-treated fetuses

In order to clarify the effects of BPA exposure on the development of neocortex, pregnant mice were treated continuously with BPA at a dose of 200 μg/kg from E8.5 (this day is the start point of mouse neocortex organogenesis). Fourteen male and 17 female fetuses from 6 litters in the BPA-treated group and 9 male and 12 female fetuses from 6 litters in the control group were sampled at E14.5 (Sup. Table 1). These fetuses were weighed and we measured the rostral-caudal length and width of the head region. The body weight and head length and width of male and female fetuses treated with BPA were comparable to those of control fetuses at E14.5 (date not shown). In addition, there was no fetal lethal effect of BPA when evaluated at E14.5 (date not shown).

As for the histopathological observation of fetuses treated with BPA, hyperplasia of the CP in the dorsal telencephalon was found in the HE staining of the parasagittal sections (Fig. 1A and B). To determine the difference of neurogenesis in fetuses treated with BPA, the Tuj1 (neuron-specific class III β-tubulin; pan-neuronal marker) expression regions were quantified by measuring the total thickness of the dorsal telencephalon and the thickness of CP (Tuj1-expression region) (Fig. 1C and D). There was a significant increase in the thickness of CP in BPA-treated fetuses (57.6 ± 2.11%, $P < 0.05$) compared with that of control fetuses (47.5 ± 2.09%) (Fig. 1E). However, hyperplasia of the CP and the expansion of Tuj1-expression area in the dorsal telencephalon were not found in the HE staining (Sup. Fig. 1A–C′) and immunostaining of Tuj1 (Sup. Fig. 1D–F′, Sup. Table 2) in the 20 μg/kg/day BPA-treated fetuses. Nakamura et al. reported that low-dose BPA might disrupt normal neocortical development by accelerated neuronal differentiation/migration (Nakamura et al., 2006). Our results coincided with these previous data and indicated that the maternal BPA oral dosing associated with the accelerated neurogenesis and hyperplasia of CP during the development of telencephalon.

3.2. Acceleration of cell cycle exit in the neural stem/progenitor cells of the dorsal telencephalon

An adequately controlled cell cycle is important for proper neurogenesis during corticogenesis in the neocortex. To investigate the cause of neurogenesis promotion, we examined cell cycle exit of neural stem/progenitor cells. We performed double immunostaining using anti-Ki67 (nucleoprotein, expressed in proliferative cells) and anti-CldU (thymidine analog) antibodies after 24 h CldU labeling (Fig. 2A and B) (Chenn and Walsh, 2002). Cell cycle exit was determined as the ratio of cells that exited the cell cycle (red, CldU+/Ki67−, indicating cells no longer dividing) to all cells labeled with CldU (red and yellow) after 24 h labeling. Quantification of the experiment showed significantly increased cell cycle exit in BPA-treated fetuses (44.1 ± 3.8%, $P < 0.01$) compared with that in the control fetuses (34.3 ± 4.7%) (Fig. 2E). However, in the 20 μg/kg/day BPA-treated fetuses, the significant increase in rate of cell cycle exit was not observed in the dorsal telencephalon (Sup. Fig. 1G–I, Sup. Table 2). These data indicated that the hyperplasia of CP was caused by the accelerated neurogenesis of neural stem/progenitor cells in the dorsal telencephalon. Ki67-expression region was decreased and the distribution of double positive cells (Ki67+ and CldU+, yellow cells) was abnormal, remaining in a deep position of Ki67-positive SVZ/VZ, compared with that in control fetuses (Fig. 2A and B). We hypothesized that these phenotypes resulted from the aberration of interkinetic nuclear migration in the SVZ/VZ, in which their nuclei migrate using radial fibers between the apical surface