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 ($En + Fn-1$).

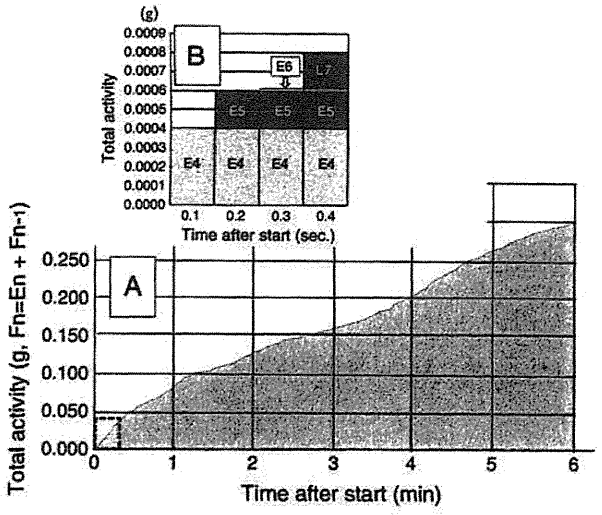

Figure 1. Total activities of intact newborn for 6 min (A) and the magnification (B, 0.4 s; see Table 1) of the area indicated by a broken line in (A).

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.

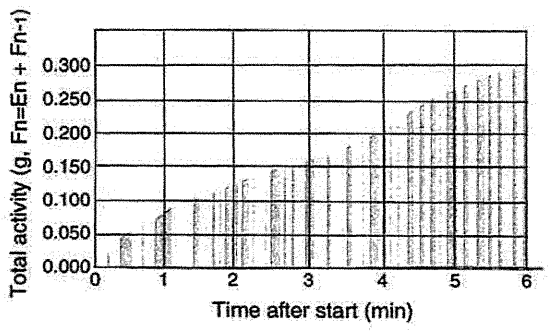

Figure 2. An integration of only absolute values showing large movements of newborn.

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

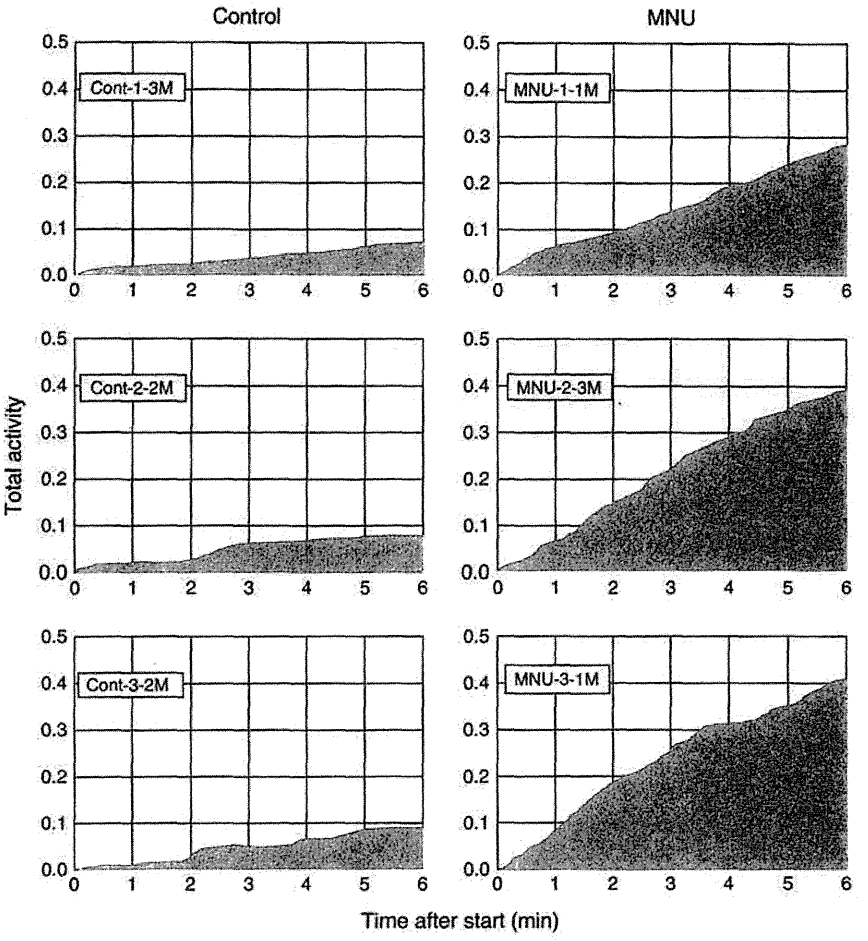

Figure 3. Representative pattern of activities for 6 min of PND 1 male newborns from three dams each in the control group and the MNU-treated group.

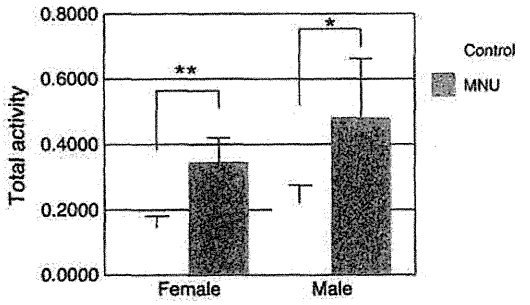

Figure 4. Mean total activities of PND 1 newborns from five dams each in the control group and the MNU-treated group. Vertical lines represent standard deviations. * Significantly different from the control, $P < 0.05$; ** significantly different from the control, $P < 0.01$.

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.

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Conflict of Interest

The authors declare that there are no conflicts of interest

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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).

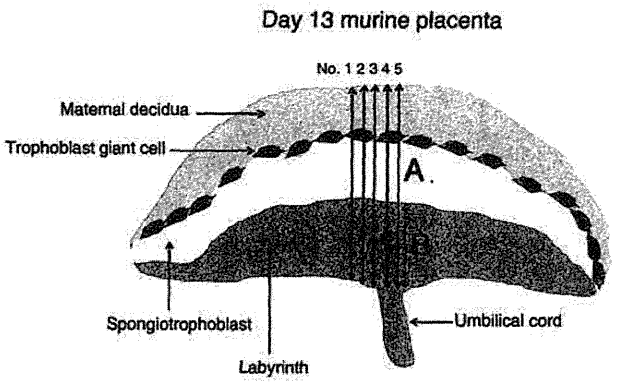

Figure 1. Diagrammatic transverse section of placenta showing the measurement of thickness. The measurement was performed with three sections close to the central portion from each placenta, and five areas (No. 1–5) close to the highest portion in each section. Fifteen values of A, B or B/A in each placenta 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.

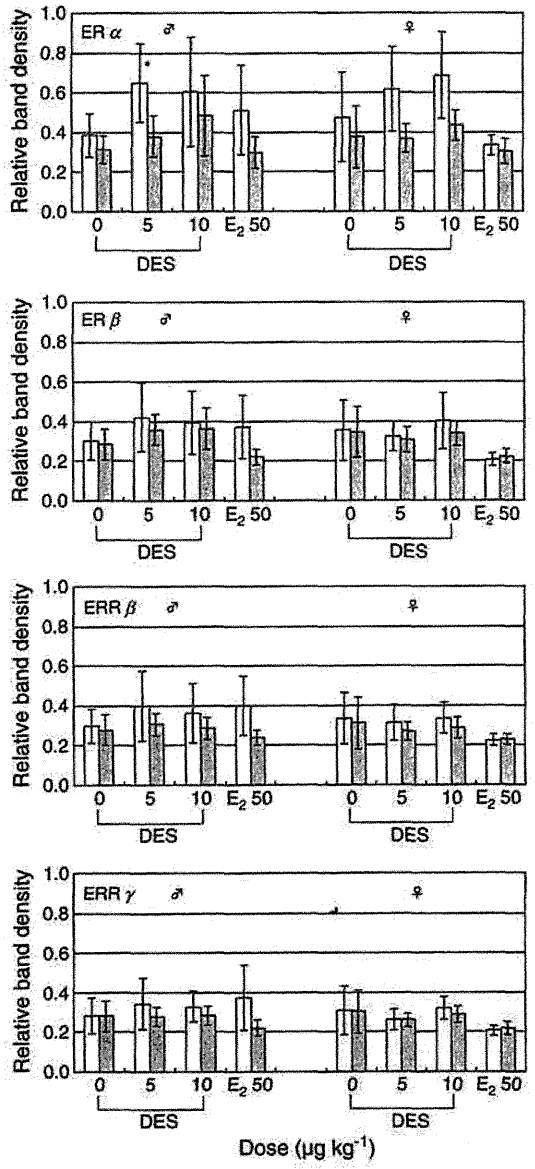

Figure 2. The expression of ERα, ERβ, ERRβ and ERRγ mRNA in the junctional zone (□) and labyrinth zone (■) of placentas with male or female embryos of dams exposed to diethylstilbestrol (DES) or 17β-estradiol (E_2) on days 4 through to 8 of gestation. *Significantly different from the control, $P < 0.05$.

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

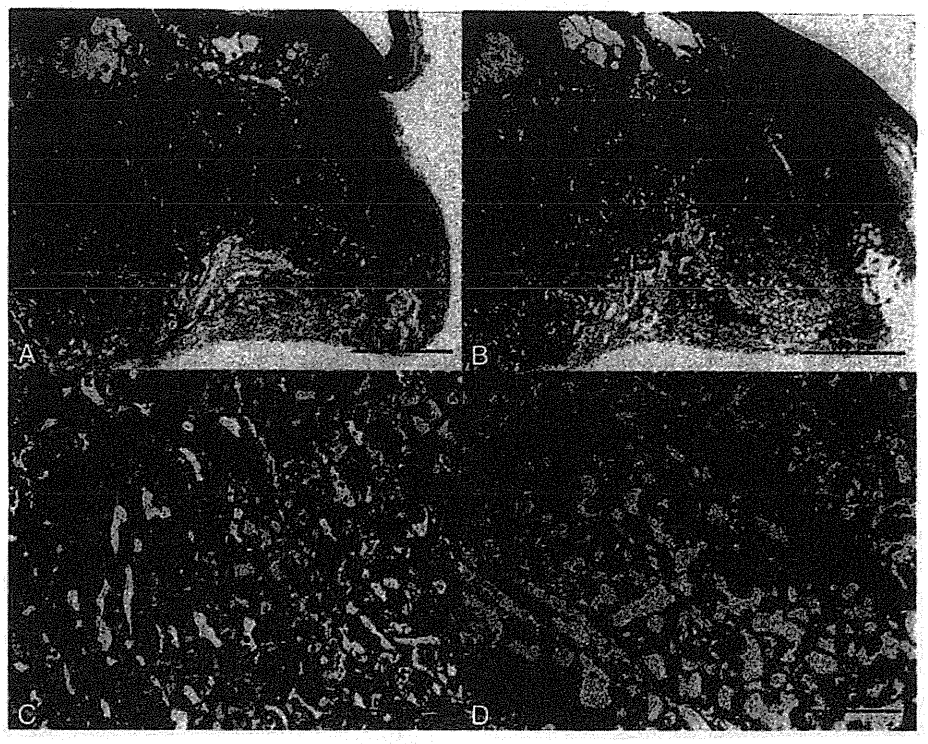

Figure 3. Light microscopic profiles of transverse sections of the control placenta (A, C) of a dam on day 13 of gestation showing a rich villous network and of the placenta (B, D) of a dam exposed to diethylstilbestrol (DES) at 10 μg $^{-1}$kg day^{-1} on days 4 through to 8 of gestation showing impaired 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.

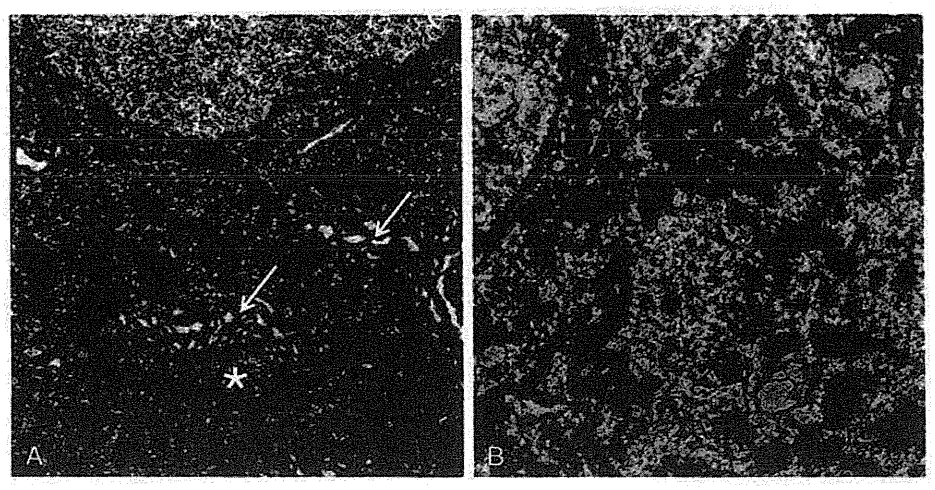

Figure 4. Electron micrographs of trophoblast giant cells in the control placenta (A) of a dam on day 13 of gestation showing rich rough-surfaced endoplasmic reticulum (*) and Golgi apparatus (↓), and in the placenta (B) of a dam exposed to diethylstilbestrol (DES) at 10 μg kg^{-1} day^{-1} on days 4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