

homologous expression system where the employed HEK293 cells endogenously expressed *Gpr30* (our unpublished data). Thus, we developed a heterologous expression system to examine whether BPA or NP could activate GPR30. For this purpose, we searched several cell types which showed no endogenous *Gpr30* gene expression, and found that a subline of RH7777 cells expressed no *Gpr30* gene. Then, we exogenously expressed FLAG-tagged GPR30 in RH7777 cells, and isolated a clonal cell line expressing GPR30, RH7777-GPR30 (Fig. 4A). For detection of GPR30 activation, [³⁵S]-GTPγS binding assay was employed, which has been established as a sensitive method detecting activation of G_{i/o} or G_s-coupled G protein-coupled receptors, including GPR30 [18,23]. When cell membranes of RH7777-GPR30 cells were stimulated by varying concentrations of BPA or NP, concentration-dependent G protein activation was observed (Fig. 4B). By contrast, there was no increase of G protein activation in cell membranes from control RH7777 cells (Fig. 4B).

Discussion

In the present study, we found that long-term exposure of fibroblasts to BPA at lower concentrations resulted in the acquisition of resistance against oxidative injury.

Similar effects were observed in fibroblasts exposed to another chemical, NP. These effects of BPA or NP were somehow in an inverted 'U' shape manner within a range of 0.1 to 100 nM. Many endocrine disruptors have been reported to show 'U' or inverted 'U' shape responses within a wide range of doses *in vivo* as well as *in vitro* [24,25].

The reasons why these non-monometric curves are observed in many experiments remain to be clarified. This may be due to the existence of two or more distinct targets for these chemicals. Alternatively, cells may intrinsically respond to maintain homeostasis when exposed to these chemicals at higher concentrations.

In addition to these non-monometric responses, in this study, the effects of BPA or NP were observed when fibroblasts were exposed to these chemicals at least one week, but not one day. Furthermore, in cell death assay, H₂O₂ was treated to fibroblasts under conditions where no chemical was exposed. These indicate that alterations of cell survival might need genomic changes during long-term exposure of fibroblasts to chemicals and that chemicals show no direct effect on H₂O₂-induced cell death. Indeed, we found that both BPA and NP could affect histone methylation, one of important epigenetic mechanisms, in 3T3 fibroblasts. Whether this epigenetic alteration directly controls bcl-2 expression would be an interesting issue to be explored.

BPA has been shown to induce various effects on many cell types *in vitro* and *in vivo*, including neuronal cells, immune cells, tumor cells, and endocrine cells [1,2]. Most, if not all, effects have been considered to be mediated via estrogenic activity through binding to nuclear or membrane receptors, including ER α , ER β , ERR γ , or

GPR30. Swiss 3T3 fibroblasts used in this study expressed only *Gpr30* gene. Thus, GPR30 is likely to be a strong candidate responsible for the increased resistance to oxidative injury in 3T3 cells exposed to BPA or NP. Indeed, BPA and NP could activate GPR30 at the concentrations which showed increased cell viability.

A several lines of evidence indicate that GPR30 couples to G_s to increase cAMP and, indeed, BPA treatment with GPR30-expressing cells results in cAMP accumulation [6,23]. The cAMP-dependent pathway-mediated cytoprotective effects have been demonstrated to likely in part involve bcl-2 expression, because cAMP responsive element (CRE) exists in the promotor region of bcl-2 gene and GPR30 activation induces bcl-2 expression [26]. Bcl-2 inhibits cell death induced by oxidative stress in many cell types, including fibroblasts [19]. Taken together, in 3T3 fibroblasts, activation of the GPR30 downstream CRE-binding protein pathway that induces bcl-2 expression might be involved in BPA- or NP-mediated increase in resistance to oxidative injury. Because BPA has been shown to influence the expression of many other anti-apoptotic or proapoptotic molecules, such as bax or caspase [27], it would be possible that these molecules are also involved in the effects of BPA or NO observed in 3T3 fibroblasts.

However, an IC_{50} for BPA to GPR30 was reported to be 630 nM [28]. By contrast, the concentrations which show cell protection and GPR30 activation were as low as 1 nM in this study. This discrepancy remains unclear. It would be speculated that long-term exposure of cells to BPA or NP at lower concentrations may result in intracellular accumulation of chemicals, then effective stimulation of G protein activity through GPR30 present in endoplasmic reticulum. Nonetheless, intracellular localization of GPR30 is still under debate, and biological significance of GPR30 in

endoplasmic reticulum remains to be determined [29,30].

Alternatively, other non-receptor mechanisms may exist, such as direct DNA insults that could cause impairment of transcription or cell cycle, leading to altered cellular responses [31]. Although we observed no changes in cell proliferation, cellular senescence involving reduced cellular responses to environmental stimuli might occur. More detail analyses will be necessary.

Here we focus on the ability of fibroblasts to resist to oxidative injury. It is unclear whether this effect on cell survival is common or limited to certain cell types. However, given that estrogen could protect neuronal cells from oxidative injury, such protective effects of BPA or NP may be commonly observed in many cell types. Therefore, the present study provide with some ideas to understand molecular mechanisms through which BPA or NP influences cellular functions.

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Figure legends

Figure 1 Swiss 3T3 fibroblasts express *Gpr30* gene. Total RNAs were extracted from cells, or mouse hippocampus (Hip) or cerebral cortex (Cor), and subjected to RT-PCR to detect genes of estrogen receptors (*Esr1* and *Esr2*), *Gpr30*, estrogen related receptors (*Esrrα*, *Esrrβ*, *Esrrγ*).

Figure 2 Long-term exposure to bisphenol A or nonylphenol ameliorates H₂O₂-induced cell death in 3T3 fibroblasts. (A) Effects of H₂O₂ treatment on 3T3 cell survival. Fibroblasts were treated with varying concentrations of H₂O₂ for two days and the number of cells were determined. Data express % of control (no H₂O₂) and are the mean ± S.D. of quadruplicates determinations. (B) Effects of 30 ~ 45 days exposure to varying concentrations of bisphenol A on H₂O₂-induced cell death. Fibroblasts were treated with 30 μM H₂O₂ for two days. Data express % of no H₂O₂ treatment in each group, and are the mean ± S.E.M. of five independent experiments. *; p < 0.05 vs no H₂O₂. (C) Effects of 30 ~ 45 days exposure to varying concentrations of nonylphenol on H₂O₂-induced cell death. Fibroblasts were treated with 30 μM H₂O₂ for two days. Data express % of no H₂O₂ treatment in each group, and are the mean ± S.E.M. of five independent experiments. *; p < 0.05 vs no H₂O₂. (D) Effects of bisphenol A or nonylphenol on bcl-2 expression in 3T3 fibroblasts. 3T3 fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP) for 30 to 40 days, and subjected to western blot assay for bcl-2 or α-tubulin. The ration of bcl-2 amounts to α-tubulin amounts was densitometrically determined. Data express % of control, and are the mean ± S.E.M. of five independent experiments. *; p < 0.05 vs DMSO. (E) Effects of bisphenol A or nonylphenol on histone H3 methylation in 3T3 fibroblasts. 3T3

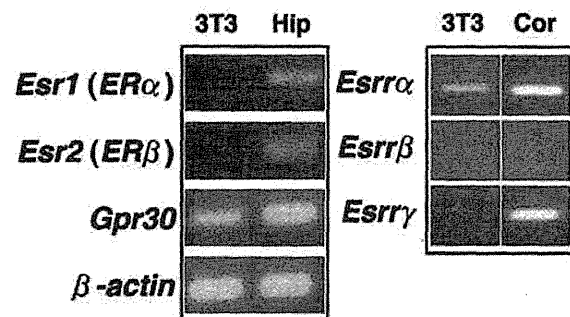
fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP) for 7 to 14 days, and subjected to western blot assay for methylated histone H3 (H3K9Me) or α -tubulin. The ratio of H3K9Me amounts to α -tubulin amounts was densitometrically determined. Data express % of control, and are the mean \pm S.E.M. of three independent experiments. *; $p < 0.05$ vs DMSO. (F) Effects of bisphenol A or nonylphenol on 3T3 fibroblast cell growth. 3T3 fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP) for 45 days, and subjected to cell growth assay. Data express as fold increase of the absorbance to values at day 0 and are the mean \pm S.D. of quadruplicate determinations from one representative experiment. (G) Phase contrast micrographs of 3T3 fibroblasts. Cells were maintained in the presence or absence of DMSO, BPA or NP for 30 days. Upper panel; cells were sparsely plated, and cultured for 1 day. Lower panels; cells are cultured for 2 days after they reached confluency. Bar indicates 78 μm for in upper panels, 250 μm in lower panels.

Figure 3 Bisphenol A or nonylphenol exposure ameliorates H_2O_2 -induced cell death in 3T3 fibroblasts cultured in D-MEM containing phenol red and fetal calf serum. (A) Effects of one month exposure to varying concentrations of bisphenol A (BPA) on H_2O_2 -induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean \pm S.E.M. from more than three independent experiments. **; $p < 0.01$, *; $p < 0.05$ vs no H_2O_2 . (B) Effects of one week exposure to varying concentrations of BPA on H_2O_2 -induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean \pm S.E.M. from more than three independent experiments. *; $p < 0.05$ vs no H_2O_2 . (C) Effects of one day exposure to

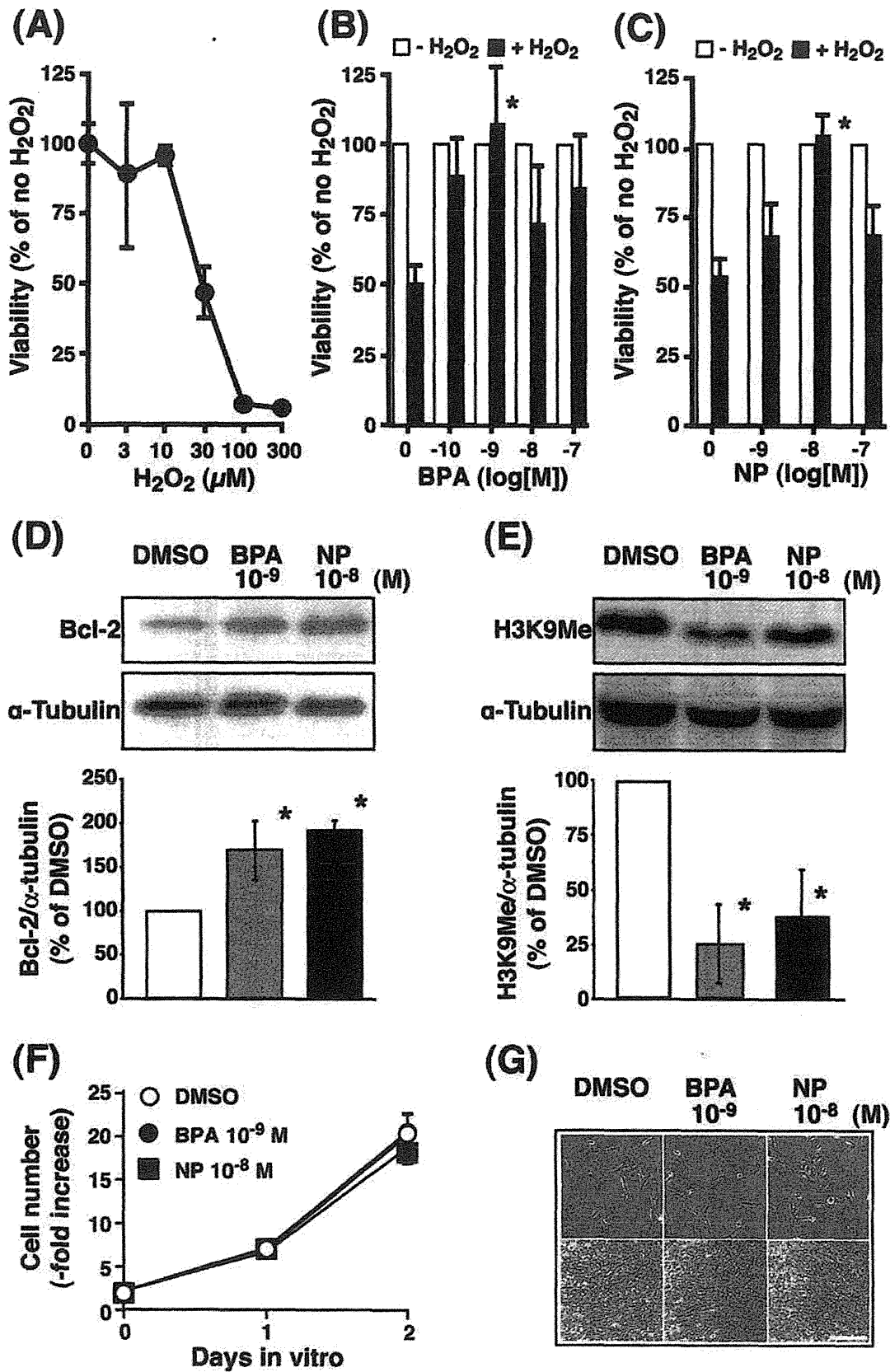
BPA on H₂O₂-induced cell death. Fibroblasts were treated with 30 μ M H₂O₂ for two days. Data express % of no H₂O₂ treatment in each group, and are the mean \pm S.E.M. of more than three independent experiments. (D) Effects of one week exposure to varying concentrations of nonylphenol (NP) on H₂O₂-induced cell death. 3T3 fibroblasts were treated with 30 μ M H₂O₂ for two days. Data express % of no H₂O₂ treatment in each group, and are the mean \pm S.E.M. of more than three independent experiments. *; $p < 0.05$ vs no H₂O₂.

Figure 4 Bisphenol A and nonylphenol induce GPR30-dependent G protein activation.

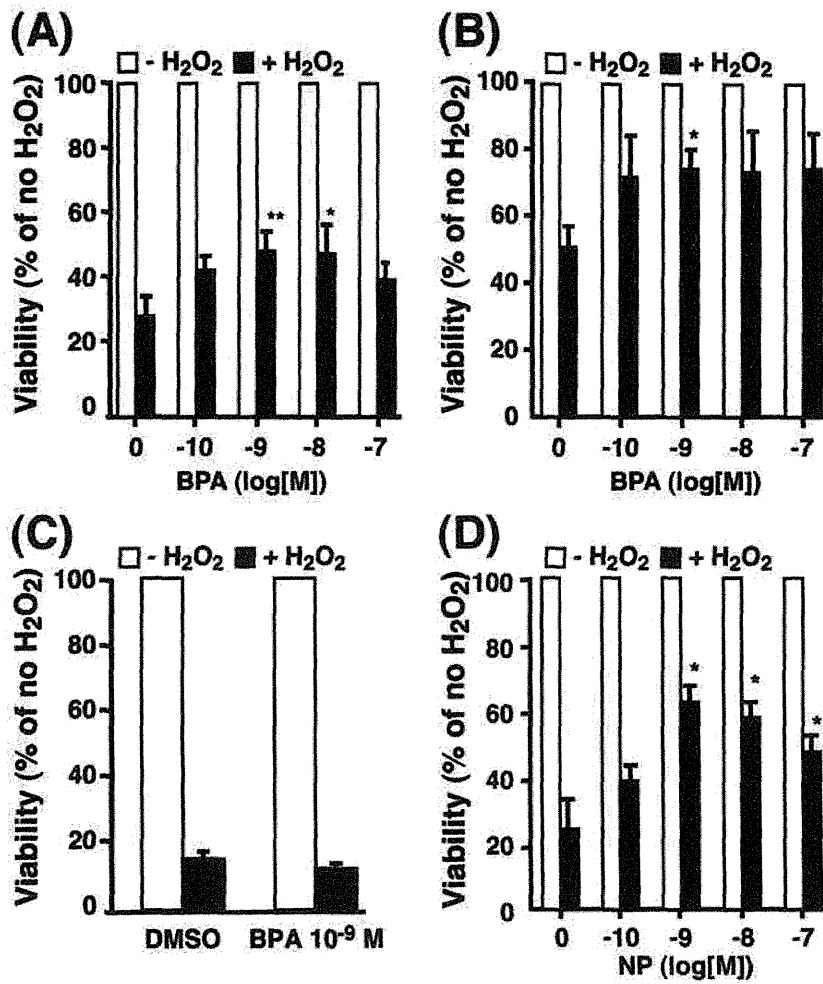
(A) Establishment of RH7777-GPR30 cell line. Total RNAs or cell membranes were prepared from control RH7777 or RH7777-GPR30 cells and subjected to RT-PCR or western blot assay using anti-FLAG antibody, respectively. (B) [³⁵S]-GTP γ S binding assay. Cell membranes were prepared from control RH7777 or RH7777-GPR30 cells and incubated with [³⁵S]-GTP γ S in the absence or presence of varying concentrations of bisphenol A (BPA) or nonylphenol (NP). The bound [³⁵S]-GTP γ S was separated and counted. Data express % of control (no drug) and are the mean \pm S.E.M. from four independent experiments. *; $p < 0.05$ vs control.



[Fig. 1 Nishimura et al.]



[Fig. 2 Nishimura et al.]



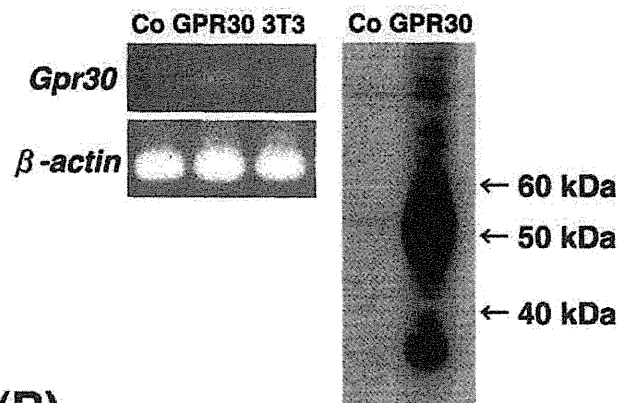
[Fig. 3 Nishimura et al.]

(A)

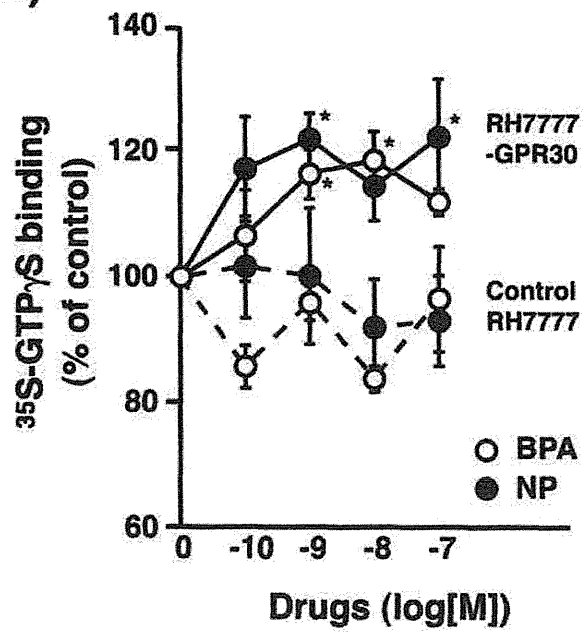


RT-PCR

Western blot



(B)



[Fig. 4 Nishimura et al.]

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SHORT COMMUNICATION

2014 in ...

Early to middle gestational exposure to diethylstilbestrol impairs the development of labyrinth zone in mouse placenta

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ABSTRACT This study was performed to clarify the involvement of impaired labyrinth zone (LZ) of the placenta in the developmental toxicity of diethylstilbestrol (DES). DES at 10 µg/kg per day was administered orally to mice on days 4 through 8 of gestation. Histological observation of the LZ and determination of blood glucose levels in dam and fetus were performed on day 13. A high frequency of embryonic death was observed in the DES group. DES induced the underdevelopment of the plexus vasculosus, extensive maternal blood space and the decreased expression of glucose transporters in the LZ, and a reduction of the glucose level in embryos. These findings suggest that impaired LZ development may be related to the embryolethality of DES.

Key Words: development, diethylstilbestrol, embryotoxicity, labyrinth zone, placenta

incidences of embryonic mortality after treatment with DES at 0, 1, 5, 10 and 15 µg/kg per day 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 per day upon oral exposure on days 4 through 8 of gestation and evaluation on day 13 in ICR mice (Nagao et al. 2010). In addition, we reported that DES affects the rough-surfaced endoplasmic reticulum of trophoblast giant cells and the developing LZ, and suggested that these developmental changes may be related to decreased protein synthesis or disruption of nutrition and oxygen exchange between mother and embryo (Nagao et al. 2010).

Thus, to address the adverse effects of oral DES administration on placentogenesis resulting in embryonic death or intrauterine growth restriction in mice, the present study was designed to examine the effects of DES administration on the development of the LZ, where most nutrient exchange between the mother and the embryo occurs.

INTRODUCTION

Placental growth and development are crucial for the development of the fetus, and dysfunction of the placenta may be closely related to fetal developmental disabilities (Bauer et al. 1998; Katayama et al. 2002; Cross 2006). The placenta is a highly vascularized tissue that develops during early gestation to facilitate the circulation of blood, oxygen, glucose and nutrients between the mother and the fetus. The rodent placenta comprises two morphologically distinct zones: the junctional zone (JZ) and the labyrinth zone (LZ) (Davies and Glasser 1968; Furukawa et al. 2011). The LZ of the placenta is the principal site of maternofetal exchange of substances. Namely, the LZ plays a role in transporting nutrients. Glucose is one of the most important substances transferred from the maternal blood to the fetal circulation in the placenta, and is a primary energy source for the fetus.

Diethylstilbestrol (DES) is a synthetic (stilbene) estrogen with an *in vivo* estrogenic potency similar to that of 17β-estradiol (E₂), and is structurally related to E₂. DES was widely used to prevent threatened miscarriages from the 1940s to 1971. In our previous study, we demonstrated that decidual hypoplasia and subsequent placental hemorrhage cause a high frequency of fetal death as a result of the oral administration of DES during the early stage of pregnancy in mice (Nagao and Yoshimura 2009). It has been shown that the

MATERIALS AND METHODS

Animals and exposure to DES

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Kinki University (Permit Number: 27–2956). ICR mice at 8 weeks old purchased from CLEA (Osaka, Japan) were used. The 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 07.00 hours) were maintained. The mice were allowed food (CE-2, CLEA, Osaka, Japan) and drinking water *ad libitum*. Ten-week-old virgin female mice were cohoused for 2 h (07.00–09.00 hours) with males aged 11 weeks or older. The females were checked for the presence of a vaginal plug immediately thereafter, and the presence of a plug represented day 0 of gestation.

Diethylstilbestrol (DES, 3,4-bis[p-hydroxyphenyl]-2,4-hexadiene) was purchased from Sigma Chemical (St. Louis, MO, USA), suspended in corn oil and administered orally on days 4 through 8 of gestation, a time in the early to middle stage of placentogenesis (Tremblay et al. 2001). Ten mice administered corn oil were used as controls. Ten mice were administered DES at 10 µg/kg per day to address the developmental effects of DES on embryo viability. Administration occurred at a defined time (12.00 hours). On day 13 of gestation, seven pregnant mice in each group were euthanized using sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) anesthesia, and the uteri from dams in each group were removed to count the numbers of live and dead embryos as well as to measure live embryonic and placental weights, and the

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determination of sex was carried out by observation of the gonadal morphology and position under a dissecting microscope. The live and dead embryos were discriminated by the onset of active heartbeat and circulation (Harris 2012). Subsequently, the placentas with live embryos were quickly washed in sterile Tyrode's solution.

Histopathological observation of the LZ

For histological observation of the LZ, two placentas per dam were selected at random. The placentas were fixed in 0.1 M phosphate-buffered 2% paraformaldehyde and 1.25% glutaraldehyde (pH 7.4). Following fixation, the placentas were rinsed three times in phosphate buffer, postfixed for 2 h at 4°C in 0.1 M phosphate-buffered 2% osmium tetroxide and dehydrated in alcohol. The placentas were embedded in epoxy resin, and semithin sections (1 µm) were stained with toluidine blue.

For observation of vascular cast in the LZ, three pregnant mice in each group on day 13 of gestation were perfused for rinsing of the blood vessels with heparinized phosphate-buffered saline (PBS) (40°C) after anesthesia using sevoflurane. Subsequently, each mouse was perfused with Mercox II resin (Ladd Research, Williston, VT, USA). The placentas infused with resin were collected and polymerized for 2 h in an oven at 60°C. Polymerized placental tissues were corroded with 20% potassium hydroxide (KOH) in the oven at 60°C for 2 days. Vascular casts were rinsed with distilled water, and the sample was dried in a desiccator. The dried specimens were affixed onto aluminum stubs with double-sided tape, sputter-coated with palladium-gold and observed under a scanning electron microscope (Hitachi S-3500N, Japan).

As for the immunohistochemical observation of glucose transporter-1 (GLUT-1) in the LZ, two placentas per dam were selected at random. The placentas were fixed for 3 h in periodate lysine paraformaldehyde (PLP) at 4°C and washed in PBS. The placentas were embedded in paraffin and sectioned at 5 µm for immunohistochemical observation. The following antibody was used: rabbit monoclonal anti-glucose transporter (GLUT-1, 1:200; Abcam, Cambridge, UK). Secondary antibody was conjugated with Alexa 488 (1:200, Invitrogen, Carlsbad, CA, USA). Immunohistochemistry was performed as described previously (Komada et al. 2012).

Measurement of blood glucose levels in dams and fetuses

The blood glucose levels in five of the seven dams in each group and their fetuses on day 13 of gestation were measured directly by a glucose oxidase method using Glucose Pilot (Aventir Biotech, Carlsbad, CA, USA) in blood samples obtained from dam and fetus, according to the manufacturer's instructions. Fetal blood was collected in 10 µL heparinized capillary tubes from the severed end of the umbilicus (umbilical vein) by the method described by Renfree et al. (1975), and maternal blood was collected from the uterine artery. Each sample from five dams and 25 fetuses per group was measured.

Statistical analysis

Mean fetal weight, placental weight and glucose level were analyzed using Student's *t*-test. The frequency of resorbed embryos was compared by the Mann-Whitney *U*-test.

RESULTS AND DISCUSSION

Embryonic mortality and the weight of embryos and placentas

No adverse effects on the general conditions in maternal animals were observed in the DES group and the control group. A significant

($P < 0.01$) increase in embryonic mortality in terms of resorptions, in the DES group (70.8%) was detected compared with that in the controls (1.9%), and the rate was compatible with that (68.5%) observed in groups exposed to DES at 10 µg/kg per day in our previous study (Nagao et al. 2010). In addition, it was reported that the embryonic mortality after oral exposure to DES at 10 µg/kg per day on days 4 through 8 of gestation (around the time of implantation to chorioallantoic fusion) was approximately 25% when examined on day 9 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 8 proceeded to death during days 9 through 13 of gestation. The trophoblast undergoes extensive villous branching to create a densely packed structure called the LZ in the mouse placenta during days 9 through 13 (Rossant and Cross 2001).

It is well known that exposure to synthetic estrogens causes a high rate of embryonic death in rodents. The administration of estradiol benzoate (EB) and DES causes a high frequency of embryonic loss in rats and mice (Scott and Adejokun 1980; Sarkar et al. 1986; Matsuura et al. 2004; Furukawa et al. 2013). Embryonic weight in both sexes in the DES group (males, 0.179 ± 0.022 g; females, 0.176 ± 0.028 g) showed a tendency to decrease compared with that in the controls (males, 0.182 ± 0.026 g; females, 0.178 ± 0.036 g), and the embryonic weight/placental weight ratio in the DES group (males, 1.761 ± 0.112 ; females, 1.739 ± 0.185) was significantly ($P < 0.05$) decreased in both sexes compared with that in the controls (males, 1.929 ± 0.212 ; females, 1.904 ± 0.233), suggesting that oral DES dosing affects the intrauterine growth of embryos.

Histopathological changes of LZ

Light microscopy of the placenta clearly showed that the control placenta of a dam on day 13 of gestation had a rich villous network in the LZ and the group exposed to DES at 10 µg/kg per day showed the lack of a developing LZ (Fig. 1A,B), and evident dilatation of maternal blood space (Fig. 1C,D). For the ultrastructural observation of the LZ, the plexus vasculosus (PV) in the LZ of the DES group was thinner than that of the control group (Fig. 1E,F), although the branching structure in the DES group was well developed, similar to that in the controls. There were no distinct differences in the development of central arterial canal (CAC) between the DES group and the control group.

The LZ, where most gaseous and nutrient exchange occurs, was markedly underdeveloped by the early to middle gestational exposure to DES. Matsuura et al. (2004) also demonstrated that the rat placenta exposed prenatally to EB lacked the developing LZ, and extensive maternal blood space was evident and the density of fetal blood vessels was significantly decreased. Furukawa et al. (2013) showed that cystic dilatation of the sinusoid was observed in the LZ of rats exposed to EB. Taking these findings together, it is suggested that disturbance of the villous network of the LZ resulting from reduced villigenesis might be associated with decreases in the physiological function of the placenta.

The cellular uptake of nutrient, glucose, across the plasma membrane is mediated by integral membrane proteins, GLUTs. Thus, the expression level of the GLUT-1 protein in the LZ of the placenta was investigated by immunohistochemical techniques. GLUT-1-positive cells in the LZ of the DES group were markedly decreased in comparison with those of the controls when evaluated on day 13 of gestation (Fig. 1G,H). Decreased GLUT-1 in the LZ of the DES group may be related to underdevelopment of the LZ, in particular, lack of a developing PV. The expression of two GLUT isoforms has been demonstrated in mammalian placenta, namely, GLUT-1 and GLUT-3 (Zhou and Bondy 1993; Devaskar et al. 1994; Barros et al. 1995). The predominant form expressed in the placenta during early

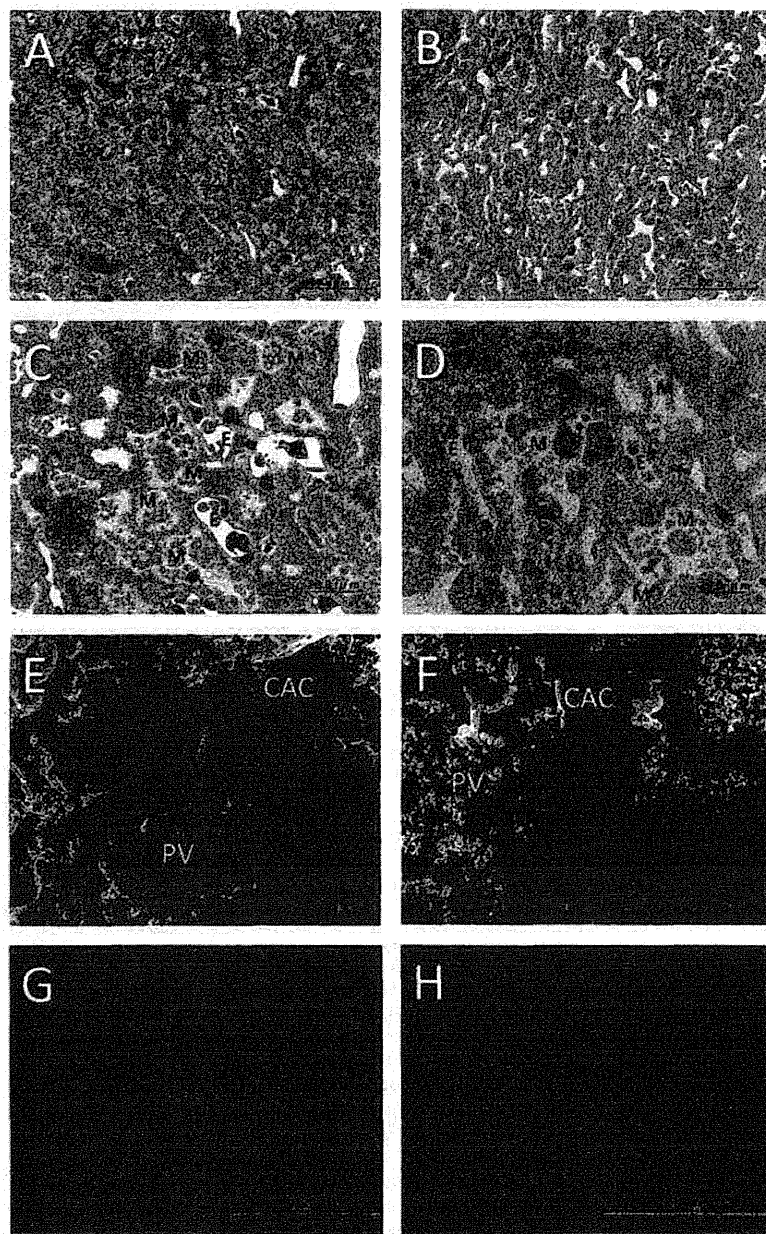


Fig. 1 Histological findings of placenta from dams on day 13 of gestation. (A,B) The labyrinth zone (LZ) of the placentas of a control dam (A) and a dam exposed to diethylstilbestrol (DES) at 10 µg/kg per day on days 4 through 8 of gestation showing impaired villous network (B). Toluidine blue staining. (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 embryo lethality 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 than 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 ($23 \pm 1^\circ\text{C}$), humidity ($50 \pm 5\%$), 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 ($D = C_n - C_{n-1}$); *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 ($E_n + F_{n-1}$; Fig. 1(B)), and total activities for 6 min ($0.1 \text{ s} \times 600 \times 6$) 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 neurotoxins 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