Developmental Toxicity of Bisphenol-A on Post-Implantation Rat Embryos Cultured *in Vitro*

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We studied the developmental toxicity and possible mechanisms of bisphenol A (BPA) damage of rat embryos *in vitro*. Whole-embryo culture was used to study the damage of BPA on visceral yolk sacs (VYSs) and embryos. Micromass culture was used to investigate the effects of BPA on differentiation and proliferation of embryonic midbrain cells. Neutral red staining was used to detect cell death. Immunohistochemistry was used to evaluate the expression of inducible nitric oxide synthase (iNOS) in VYSs and embryos. Our results as following, BPA could cause damage to embryonic development and morphological differentiation in a dose-dependent manner. At the highest dose, 80 and 100 mg/l BPA delayed cardiac tube growth and differentiation of VYSs, and induced various embryonic defects. BPA also induced excessive cell death and inhibited both proliferation and differentiation of rat midbrain cells. Immunohistochemical staining showed that BPA induces abnormal expression of iNOS protein in treated VYSs and embryos. Our results indicated that BPA is a potential teratogen and has toxicity on cultured rat embryo development. BPA-related developmental toxicity is caused by damage to the VYS, excessive cell death, inhibition of cell proliferation and differentiation, and abnormal expression of iNOS.

Key words —— bisphenol A, whole-embryo culture, developmental toxicity, micromass culture, nitrogen monoxidum

INTRODUCTION

In recent years, the association between the alterations in animal hormonal regulation and exposure to endocrine-disrupting chemicals, such as xenoestrogens, has led to increasing public and scientific concerns.[1)] Among the xenestrogens, bisphenol A (BPA, $C_{15}H_{16}O_2$, Fig. 1), an estrogen-activity compound, has received much attention.[2)] BPA is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins that are extensively used in the interior coating of cans in the food-packaging industry and in dentistry.[3)] BPA has two unsaturated phenol rings and shares similarity with synthetic estrogens such as diethylstilboestrol, hexoestriol, and the bisphenolic component of tamoxifen. BPA has little structural homology with estradiol. Both *in vitro* and *in vivo* short-term assays have shown that BPA has weak estrogenic activity.[4,5)] The treatment of omariectomized (OVX) Wistar rats with BPA induces uterine growth and stimulates significant growth of the anterior pituitary gland in a dose-dependent manner.[6)] People who are exposed to BPA through their respiratory tract, alimentary tract and skin can experience extensive damage to their kidneys, liver, spleen, pancreas, and lungs.[7)] Previous studies have proven that BPA has potential genetic toxicity and embryotoxicity;[8,9)] however, the mechanism of BPA development toxicity is unclear. We explored the mechanism of BPA teratogenicity by using whole-embryo culture and micromass culture and confirmed that BPA is toxic to embryo development.

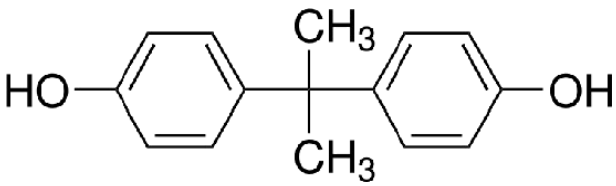

Fig. 1. Molecular Structure of Bisphenol A

MATERIALS AND METHODS

Chemicals —— BPA (>99% purity) and Hams' F12 media was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Monoclonal inducible ni-

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tric oxide synthase (iNOS) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Immunhistochemical kits were obtained from Boster Company (Wuhan, China). Trypsin was purchased from Hyclone Co. (Logan, UT, U.S.A.). All chemicals used were of analytical grade.

Animals —— The study protocol was approved by Taishan Medical University. Wistar rats used in this study were obtained from Vital River Lab Animal Technology Co. Ltd., Beijing, China. Each male was caged overnight with two nulliparous females. Pregnancy was confirmed the following morning by the presence of a vaginal plug; this was considered as gestational day zero (GD0). At GD9.5 and GD13, pregnant rats were sacrificed by cervical dislocation and their uteri were removed for the preparation of embryonic cultures. The females are considered 0.5 days pregnant at noon on the day of sacrifice as copulation was assumed to have occurred within 2 hr of midnight.

Rats were housed under controlled conditions at 23 ± 1°C, 40–60% humidity, and a 12-hr light/dark cycle. Rats were fed an *ad libitum* solid diet with tap water. The use of animals was conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology (adopted by the Society of Toxicology in July 1989, revised March 1999).

Whole-embryo Culture —— Embryos were explanted according to the method developed by New and adapted by Liu *et al.*[10, 11] Of the GD9.5 embryos, those displaying 3–5 pairs of somites were selected for culture. After removal of Reichert's membrane, those embryos with an intact yolk sac and ectoplacental cones were placed in sealed culture bottles (3 embryos per bottle) containing 3 ml of culture medium. Cultures were incubated at 37°C and bottles were rotated at 35 rev/min. Culture medium was rat serum (from healthy adult Wistar rats weighing 250–280 g) that was immediately centrifuged, heat-inactivated (56°C for 30 min), filter sterilized, and supplemented with 100 units/ml penicillin G (Sigma) and 100 μg/ml streptomycin. BPA powder was dissolved in dimethyl sulfoxide (DMSO) and added to culture medium to gain the final concentration of 20, 40, 60, 80, 100 mg/l. Control cultures received DMSO only. The culture medium was initially treated for 2.5 min with 5% O_2, 5% CO_2, 90% N_2. Subsequent treatments for another 2.5-min duration occurred at 16 hr (20% O_2, 5% CO_2, 75% N_2) and 26 hr (40% O_2, 5% CO_2, 55% N_2). All embryonic rat cultures were terminated at 48 hr and the embryos were evaluated for viability by the presence of yolk sac circulation and heartbeat. Viable embryos were assessed for development by the morphological scoring system developed by Van *et al.* (1990).[12]

Histological Detection —— Immediately after we measured and assessed the embryos, some living embryos were stained using a neutral red staining protocol[13] to detect cell death in whole-mounted tissues. Other embryos were immediately immersed in 3% glutaraldehyde, and prepared for scanning and/or transmission electron microscopy (SEM and TEM). The remaining embryos and visceral yolk sacs (VYSs) were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in paraffin. These embryos were serially sectioned in 4-μm slices. Some sections were stained with hematoxylin-eosin (H&E) to evaluate the difference between the control and treated embryo tissues.

Immunhistochemistry —— Deparaffinized and dehydrated sections were immersed for 10 min in 0.3% hydrogen at room temperature to destroy endogenous peroxidase activity. The sections were then rinsed for 10 min with phosphate-buffered saline (PBS), and then incubated for 20 min with 10% normal goat serum at room temperature. Antibody binding was performed following the immunohistochemical kit manufacturer's instructions. The detection was carried out with 3,30-diaminobenzidine (DAB) followed by washing and staining with Mayer's hematoxylin. Control sections were labeled with non-specific IgG antiserum.

Localization of antigen-antibody complexes was qualitatively evaluated by the density of peroxidase reaction product, with relative intensity rated on a scale of 1–4. Evaluations were performed on ten serial sections from four different embryos per treatment group and representative sections were photographed.

Micromass Culture —— Rat embryo midbrain mesenchymal cells were cultured according to Flint and Orton.[14] Midbrains were removed from GD13 rat embryos and trypsinized to prepare single-cell suspensions in Ham's F12 culture media containing 20% fetal bovine serum, 548.6 mg/l L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cell density was adjusted to 5×10^6 and the samples were treated with a range of BPA concentrations (0, 10, 20, 30, 40, 50 mg/l). To assess differentiation, 20 μl of cell suspension was plated on pre-warmed (37°C) 24-well plastic culture dishes. To access cy-

totoxicity, a 10-μl droplet of each well was plated into wells of a pre-warmed (37°C) 96-well culture plate.

Hematoxylin staining was used as an indicator of neuronal differentiation of midbrain cells cultures. After being cultured for 5 days, the medium was removed and cell cultures were fixed with 2.5% (v/v) formaldehyde for 20 min. Cells were then rinsed with tap water and treated with Mayer's hematoxylin for 3–4 min. The intensity of hematoxylin staining in the midbrain cell cultures is greatest in areas of the cultures where there are large numbers of neuronal cells.

Cytotoxicity was quantified in the midbrain cell cultures using the methylthiazoltetrazolium (MTT) assay. After being 5 days in culture, wells were treated with MTT solution at 5 mg/ml. Cells were incubated for 4 hr with MTT at 37°C and 10% CO_2. The solution was subsequently removed by aspiration and replaced by 100 μl of DMSO per well to dissolve the otherwise insoluble purple MTT-formazan product. Sample absorbance was measured at 570 nm in the linear range of the absorption curve of an Anthos Labtec reader.

Statistics —— All data are expressed as means ± S.D. (standard deviation). Differences between groups were analyzed by using an analysis of variance (ANOVA) followed by either the least significant difference (LSD) post-hoc test or Dunnett's T3 post-hoc test. The incidence of abnormalities was compared using a chi-square test with continuity correction. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the statistical software package SPSS 11.0 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

The Toxicity of BPA on the Development of Cultured Rat Embryos

Rat embryonic growth and morphology were examined after 48 hr in culture. Control embryos exhibited normal growth and development (Fig. 2A) with normal yolk-sac circulation. Cell death was rare in control embryos with only hearts and forelimbs slightly stained by neutral red (Fig. 3A). Continuous exposure to BPA for 48 hr decreased yolk sac diameter, somite number, crown-rump length, head length, and developmental scores, but increased the percent of abnormal embryos (Table 1). At 60 mg/l, the effect on growth was significantly reduced compared to control ($p < 0.05$). There was also a dose-related trend for each parameter. The development score was significantly reduced at 60 mg/l compared to control and displayed a concentration-dependent score. Embryos exposed to BPA also showed a concentration-related trend for malformation incidence. 100 mg/l BPA produced abnormalities in 100% of the embryos and produced one dead embryo. These results indicate that BPA caused retarded growth of embryos and inhibited the differentiation of embryonic organs. Abnormalities observed in BPA-treated embryos were primarily delayed development, an open neural tube, hydrocephaly, abnormal brain development, abnormal heart and flexion, and small forelimb bud formation. Increasing the concentration of

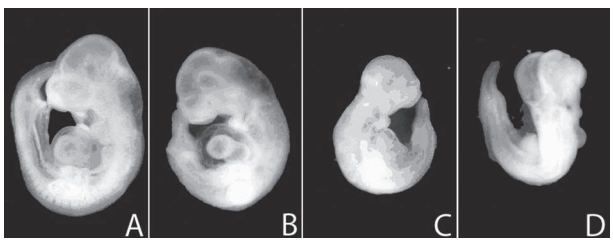

Fig. 2. GD11.5 Rat Embryos Exposed to BPA for 48 hr

(A) Normal GD11.5 embryo. (B) Embryo exposed to 60 mg/l BPA, pericardial edema. (C) Embryo exposed to 80 mg/l BPA, abnormal fourth ventricle of cerebrum. (D) Embryo exposed to 100 mg/l BPA, open neural tube and abnormal flexion (×12).

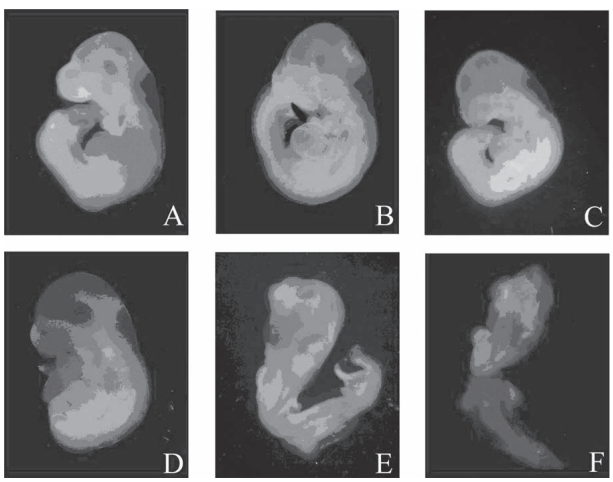

Fig. 3. GD11.5 Rat Embryos Were Stained by Neural Red after Exposed to BPA for 48 hr

A–F: embryos exposed to 0, 20, 40, 60, 80, 100 mg/l BPA, respectively. Cell death was rare in control embryos treated with either 20 mg/l (B) or 40 mg/l (C) BPA — only the heart and forelimb were slightly stained by neutral red. Embryos exposed to 60 mg/l (D), 80 mg/l (E) and 100 mg/l BPA (F) had more cell death compared to control embryos.

Table 1. Effects of BPA Exposure on Rat Embryos after 48 hr of Culture

Concentration (mg/l)	Number of embryos	Yolk sac diameter (mm)	Crown-rump length (mm)	Head length (mm)	Pairs of somites	Morphological score[a)]	Number of retarded growth embryos	Number of malformations embryos[b)]
0	13	4.86 ± 0.05	4.04 ± 0.11	2.04 ± 0.11	29.0 ± 1.0	31.8 ± 0.4	0	0
20	9	4.80 ± 0.30	3.81 ± 0.31	1.91 ± 0.18	27.3 ± 1.5	31.3 ± 0.7	0	0
40	10	4.60 ± 0.39	3.81 ± 0.30	1.94 ± 0.30	27.1 ± 1.4	30.9 ± 1.5	1	0
60	9	4.14 ± 0.20*	3.73 ± 0.21*	1.77 ± 0.06*	25.3 ± 1.2*	25.7 ± 5.9*	0	3
80	8	3.59 ± 0.20*	3.06 ± 0.40*	1.53 ± 0.24*	19.5 ± 7.6*	19.8 ± 5.7*	2	5
100	10	3.16 ± 0.18*	2.59 ± 0.26*	1.21 ± 0.08*	17.4 ± 4.0*	18.1 ± 2.7*	0	9

Morphological features were scored by Li Yong. *a*) Based on yolk sac circulation, flexion, nervous system, visual system, olfactory system, heart, forelimb bud and tail. *b*) Chi-square for trend: $\chi^2 = 13.94$, $p < 0.001$. * Compared with control, $p < 0.05$.

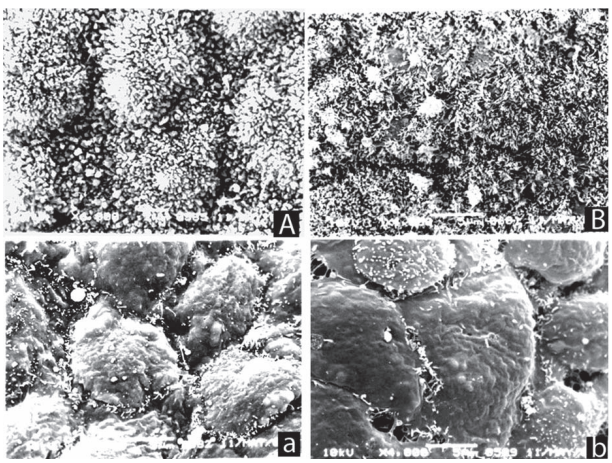

Fig. 4. Ultrastructure of the VYS Following Exposure to 0 or 80 mg/l BPA for 48 hr

A, Endodermis cells as controls (×6000); B, 80 mg/l BPA (×4000); a, Mesothelium cells: control (×4000); b, 80 mg/l BPA (×4000). Compared to control, the ultrastructure of VYS exposed to 80 mg/l BPA is abnormal under light microscopy. Three layers of the structure were reduced in thickness. The number of small vessels and red blood cells decreased or vanished completely; numbers of microvilli, lysosomes, mitochondria and endoplasmic reticulum also decreased. No regular microvilli or swollen mitochondria were found.

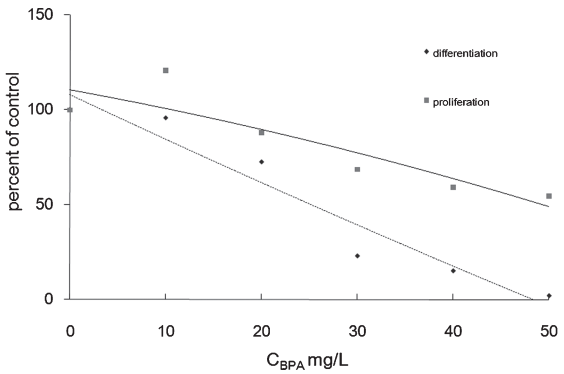

Fig. 5. Effect of BPA on the Cytotoxicity and Differentiation of Rat Embryonic Mid-brain Cells

Embryonic cells were isolated from rat embryos on GD13 and cultured for 5 days. Cytotoxicity and differentiation were determined as described in Sec. 2. Each point represents the mean ± S.D. of two experiments, conducted in twelve and eight replicate wells per experiment, respectively. Cytotoxicity and differentiation data were well-described by equations for a straight line.

BPA also enhanced the incidence of cell death and teratosis (Fig. 3).

Even when the BPA concentration was not less than 60 mg/l, the ultrastructure of the VYS was clearly abnormal under light microscopy. The number of small vessels and red blood cells decreased or vanished and the number of microvilli, lysosomes, mitochondria, and endoplasmic reticulum also decreased. No regular microvilli or swelling mitochondria were found (Fig. 4).

Effects of BPA on Cultured Midbrain Cells

Micromass cultures of rat embryonic midbrain cells exposed to BPA showed dose-dependent reduction in both total cell number and differentiation into neurocytes. However, the latter endpoint was more sensitive. In control cultures, the cells proliferated, and pre-neurocytic cells condensed into microscopically observable foci that stained upon exposure to hematoxylin. In 20 mg/l BPA-treated cultures, the number of foci per spot culture was significantly diminished compared to the control. The estimated concentration of BPA required for a 50% reduction in the number of foci compared to the controls was 24.58 mg/l (Table 2 and Fig. 5).

Cell proliferation, as measured by MTT assay, was significantly reduced at 30 mg/l BPA. Measured as a percentage relative to untreated controls cells, viability decreased with increasing BPA concentration in a dose-dependent manner; approximately 57.20 mg/l BPA was needed to reduce cell viability by 50% (Table 2 and Fig. 5).

Table 2. Influence of BPA on the Differentiation and Proliferation of Rat Embryo Midbrain Cells

Concentration (mg/l)	Differentiation			Proliferation		
	N	Number of foci ($x \pm s$)	Percent of control	*N*	OD ($x \pm s$)	Percent of control
0	8	99.3 ± 12.7	100.0	12	0.267 ± 0.041	100.0
10	4	95.0 ± 19.5	95.7	8	0.322 ± 0.043	120.9
20	4	72.0 ± 13.1*	72.5	8	0.235 ± 0.010	88.2
30	4	22.8 ± 8.1**	23.0	8	0.183 ± 0.011**	68.7
40	4	15.0 ± 5.9**	15.1	8	0.158 ± 0.025**	59.3
50	4	1.0 ± 2.0**	2.0	8	0.146 ± 0.011**	54.6

Compared with control: $^{*}p < 0.05$, $^{**}p < 0.01$.

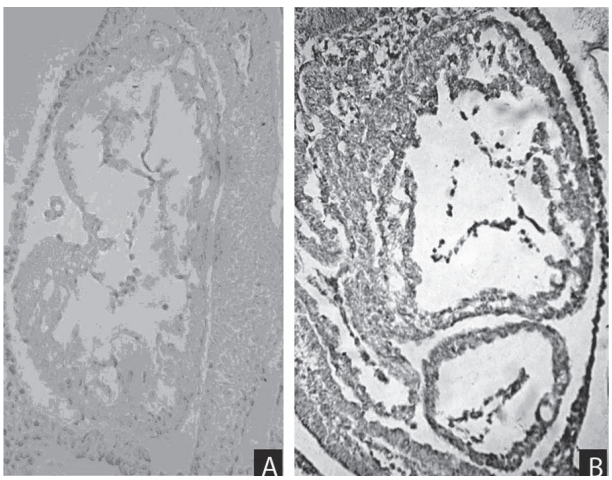

Fig. 6. GD11.5 Rat Heart Was Stained for iNOS

A: control embryo heart demonstrating a pale immunolabeling (1+); B: BPA-exposed heart demonstrating a dense reaction production (4+) over the cytoplasm of mesenchymal cells and epitheliums (× 50).

Expression of iNOS in VYS and Embryos

To investigate the mechanism through which BPA may act on embryo development, we performed immunohistochemistry to determine the expression of iNOS in BPA-treated embryos and VYSs. The distribution of iNOS was examined in both BPA-treated and untreated GD11.5 embryos. Control embryos rarely exhibited immunolabeling (1+) for the presence of iNOS (Fig. 6A). In contrast, the mesenchyme of BPA-exposed embryos was densely labeled (4+) for the presence of iNOS (Fig. 6B).

DISCUSSION

The teratogenic potential of BPA has had conflicting results in *in vivo* studies. Hardin *et al.* (1981)[15] administered BPA by intraperitoneal injection to Sprague-Dawley rats on GD1–15 at doses of 85 or 125 mg/kg per day and found that either dose caused a significant reduction in the number of live fetuses per litter and dose-dependent decreases in fetal body weight and crown-rump length. In addition, in the high-dose BPA-treated group, they found an increase in litters with fetuses having enlarged cerebral ventricles or hydrocephaly. Morrissey *et al.*, however, found that post-implantation exposure to BPA by oral gavage did not cause external, visceral, or skeletal malformations at doses that caused significant maternal toxicity in rats or mortality in mice.[16] It was also reported that BPA could induce teratosis formation of rat.[17] Similarly, we find that high-levels of BPA *in vitro* can influence cell growth and morphological differentiation resulting in malformed embryos with hydrocephaly, small forebrain and midbrain, open neural tube, abnormal heart, small forelimb bud and abnormal optic, and abnormal flexion. These irregularities clearly demonstrate that BPA is developmentally toxic to rats.

The VYS has many important physiological functions in the early development of rodents, including nutrition and metabolism, and is the only functional placenta at the early embryo morphodifferentiation stage [*i.e.* until embryonic day (E) 12.5 in mice]. Any damage to the function and structure of the VYS at this stage would influence the normal development of embryos, induce teratogenesis, or even kill the embryos.[18, 19] Our results show that high levels of BPA inhibited the growth and development of the VYS. At 60 mg/l BPA, the diameter and vessel differentiation score of the VYS were decreased. Furthermore, the VYS ultrastructure was significantly changed, showing signs of endocrine cell denaturation, necrosis and mitochondrial swelling, which influence digestion, phagocytosis and secretion. We determined the num-

ber of microvessels, red blood cells and blood islands by electron microscopy and noted a reduction in all three. The reduction reflected an abnormality in blood vessel differentiation and changes in hematopoietic function leading to oxygen deprivation and lack of nutrition during normal embryo development. The changes also resulted in embryonic hypoxia and metabolic turbulence, inducing abnormal embryonic development. The results indicate that VYS is an important "target" site of BPA, and that the damage to VYS structure and function might be one possible mechanism of BPA's developmental toxicity.

Cell death has been shown to play an important role in many morphogenetic processes during embryonic development and has been well studied during early development of limb buds,[20] the heart,[21] the somite,[22] the tail bud,[23] and the developing nervous system.[24] In the present study, some of the organs from untreated control embryos, including hearts, tails, forelimbs and somites, were slightly stained by neural red, demonstrating that these developmental tissues exhibited physiological or programmed cell death. BPA-treated embryos showed increased cell death in the brain, branchial arches, hearts, somites and forelimbs compared to control embryos. Interestingly, BPA most induced cell death in regions with morphological abnormalities. Excessive cell death in the brain might contribute to the lack of neural tube closure and hydrocephaly. Induction of pericardial edema and developmental retardation of the heart may contribute to increased cell death in the heart. The results of our histological detection and electron microscopy indicate that there was cell death occurring in the embryonic tissues and VYSs, but the specific cells undergoing apoptosis were not identified. However, our results demonstrate that BPA can induce cell death, which may affect embryonic development.

Normal embryo development is influenced by many factors whose perturbation can lead to embryonic defects. Differentiation and proliferation of cells are key processes in embryo development; inhibition of these processes can certainly disturb embryonic development.[14, 25] Gursoy *et al.* found that activated BPA in cultured HT-22 cells could exert protective effects against neurotoxins,[26] which indicated that BPA could be activated in neurocytes. Another experiment showed that BPA could disturb the neuro-endocrine axis if given during a period critical for brain organization (*i.e.*, from day 14 of gestation to 6 days after birth), resulting in altered nonsocial behavior in offspring.[27] In the present study, our results with micromass culture show that BPA inhibited the differentiation and proliferation of cultured midbrain cells. At high levels, BPA was cytotoxic to cultured midbrain cells of the Wistar rat embryo and also inhibited clone formation, which proved that BPA could inhibit the formation of neurocytes in the brain. Although little is known about the mechanism by which BPA inhibits midbrain cells, experiments by Atkinson have demonstrated that *in vitro* incubation of DNA with BPA in the presence of a peroxidase activation system could produce one major and seven minor DNA adducts.[28] In addition, Tsutsui *et al.* found that BPA induced aneuploidy in cultured Syrian hamster embryo cells and Pfeiffer found that BPA was a potential aneuploidogen.[29, 30] These results might give us some clues as to the mechanism of the inhibition of midbrain cells by BPA. The inhibition of proliferation and differentiation of the midbrain might contribute to the developmental toxicity seen with BPA.

Noguchi *et al.* demonstrated that BPA (1–100 μM) increased the levels of nitrite/nitrate, which are stable metabolites of NO in culture medium of MSS31.[31] Immunohistochemistry showed BPA-treated embryos and BPA-treated VYSs express abnormal iNOS compared to controls, which might be another mechanism of BPA action.

Nitric oxide (NO) is an important second messenger. NO is not only involved in natural biochemical and physiological processes, but may also be involved in embryonic development and teratogenesis. iNOS is regarded as an effective biomarker in the screening of developmental toxicants in teratological research. Nitric oxide synthase (NOS) is a multifunctional and widely distributed isoenzyme that plays an important role in a series of tissue and organs.[32, 33] There are three isoforms of NOS: endothelial, inducible and neuronal. The endothelial and neuronal isoforms are activated by intracellular calcium and are constitutively expressed, whereas iNOS is not expressed during normal development. When cells respond to abnormal conditions, the expression of iNOS is induced, which results in large quantities of synthesized NO from its precursor L-arginine.[34, 35] Increasing NO can interfere with growth and development of embryos in multiple ways. By virtue of its unpaired electron, NO is a free radical that can react with molecular oxygen and produce two other free radicals, hydroxy and nitrogen dioxide. These free radicals can influence the process of development through lipid

peroxidation. NO can interact with mitochondrial enzymes and inhibit respiration of the VYS and cellular mitochondria of the embryo. NO inhibits DNA replication enzymes, which can interrupt DNA synthesis in replicating embryonic cells. High doses of NO can be directly cytotoxic to embryos or VYS cells.

In conclusion, BPA demonstrates developmental toxicity to rat embryos *in vitro*. It directly damages embryos, induces cell death, and inhibits cell proliferation and differentiation. Moreover, BPA can damage VYS structure and function and can cause abnormal expression of iNOS in the VYS and embryonic cells, which might be a potential mechanism for its developmental toxicity in cultured rat embryos.

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