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# Assessment of Bisphenol A (BPA) neurotoxicity *in vitro* with mouse embryonic stem cells

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ABSTRACT

The adverse effects of environmental pollution on our well-being have been intensively studied with many *in vitro* and *in vivo* systems. In our group, we focus on stem cell toxicology due to the multitude of embryonic stem cell (ESC) properties which can be exerted in toxicity assays. In fact, ESCs can differentiate in culture to mimic embryonic development *in vivo*, or specifically to virtually any kind of somatic cells. Here, we used the toxicant Bisphenol A (BPA), a chemical known as a hazard to infants and children, and showed that our stem cell toxicology system was able to efficiently recapitulate most of the toxic effects of BPA previously detected by *in vitro* system or animal tests. More precisely, we demonstrated that BPA affected the proper specification of germ layers during our *in vitro* mimicking of the embryonic development, as well as the establishment of neural ectoderm and neural progenitor cells.



## Introduction

During our everyday life we are constantly exposed to many artificial substances created in numerous industrial processes. Many of these materials tend to accumulate in the environment. As a consequence, human exposure to these chemicals, and the potential adverse health effects caused by them, may occur even in the absence of direct use of these industrial products. This has raised a growing concern about the effects of environmental pollution on human health and prompted the urgency and necessity of validated and comprehensive toxicity tests to address the potential hazardousness of these pollutants. Stem cell toxicology is a very powerful alternative to animal tests or traditional *in vitro* assays because it allows to test *in vitro* the acute and developmental toxicities of a pollutant of interest, quickly, thoroughly, and cost-effectively (Faiola et al., 2015; Jennings, 2014). In fact, embryonic stem cells (ESCs) offer the advantage that they can be derived easily and also cultured indefinitely in dishes. Therefore, they can be employed without problems for cytotoxicity assays like any other kind of cells. In addition, ESCs can be utilized in developmental toxicity assays. For instance, they can differentiate *in vitro* as three dimensional aggregates so-called embryoid bodies (EBs) which mimic the early stages of embryonic development *in vivo*. Moreover, ESCs can virtually differentiate specifically into any type of cells of an adult organism. Those cells can then be used

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for cell function toxicity assays as any other derived primary cell type (Wobus and Loser, 2011; Liu et al., 2013; Mori and Hara, 2013).

One of the most studied and dreaded environmental pollutants to date is a chemical called Bisphenol A (BPA). It has been employed since the 1950s to make polycarbonate plastics in a variety of products. BPA has been also used to coat the inner surface of beverage and food containers, regardless of the fact that it has been recognized as an endocrine-disrupting chemical (EDC) since 1936 (Dodds and Lawson, 1936). Indeed, countless studies in the last two decades have documented BPA-related effects on fertility, genital and behavioral abnormalities, heart disease, diabetes, and obesity (Rochester, 2013). However, in those reports, many different *in vitro* and *in vivo* systems have been employed, but almost none utilized stem cells. In addition, when BPA was tested with mouse ESCs, little or no toxic effects were detected (Panzica-Kelly et al., 2013; Kong et al., 2013).

In this study, we employed the known developmental toxicant BPA to test its effects on mouse ESCs with our stem cell toxicology system. Contrary to previous reports, we were able to detect BPA toxicity *in vitro*, particularly towards the neural ectoderm specification.

## 1. Materials and methods

### 1.1. Cell culture

All cell culture reagents and plasticware were purchased from Gibco (Life Technologies, NY, USA) and Corning (USA), respectively, unless otherwise indicated. J1 mouse ES cells were acquired from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured at 37°C in humidified air with 5% $CO_2$ in high glucose KnockOut DMEM medium supplemented with 15% fetal bovine serum, 2% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), 1% nucleosides, 1% glutamine, 1% non-essential amino acids, $10^{-4}$ mol/L β-mercaptoethanol and $10^3$ U/mL leukemia inhibitory factor (LIF) (Merck Millipore, Darmstadt, Germany). All plates were coated with 0.1% gelatin (Sigma-Aldrich, MO, USA) before use.

### 1.2. Cytotoxicity assay

For cytotoxicity assays, mESCs were pre-incubated with 1–10 μmol/L BPA (or DMSO solvent control) for 24 hr, then seeded in 96-well gelatin-coated plates, and incubated with BPA/DMSO for seven days. Cell viability was determined by the AlamarBlue (AB, Sigma-Aldrich, MO, USA) fluorescence assay. Briefly, cells were incubate with the AlamarBlue reagent for two hours at 37°C, and fluorescence was measured in a multiwall fluorometric reader (ThermoFisher Scientific, MA, USA) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

### 1.3. Alkaline phosphatase (AP) staining

To visually detect undifferentiated mESCs, an AP staining kit (Sigma-Aldrich, MO, USA) was used according to the manufacturer's instructions.

### 1.4. Embryoid body (EB) differentiation assay

For EB differentiation assays, $4 \times 10^6$ mESCs were resuspended by trypsinization and seeded in 10-cm petri dishes in ES medium without LIF, to allow aggregation and formation of EBs. Media were replaced every other day and samples collected at days 0, 2, 4, 6, 9, 12, and 20, for RNA extraction and qRT-PCR measures. One day before the start and along the differentiation process, cells were incubated with 10 μmol/L BPA or DMSO control.

### 1.5. mESC differentiation into neural progenitor cells (NPCs)

mESCs were pre-treated with 10 μmol/L BPA or vehicle control for 24 hr, and then allowed to form EBs as described above. At day 4 of EB formation, a final concentration of 5 μmol/L retinoic acid (RA, Sigma-Aldrich, MO, USA) was added to the medium to stimulate neural ectoderm specification. At day 8, EBs were dissociated into single cells by trypsinization and passage through a 40 μm nylon cell strainer (BD, USA), and seeded into plates coated with laminin/poly-L-lysine (Roche, USA, and Sigma-Aldrich, USA, respectively) in N2 medium (DMEM-F12, N2 supplement, 1% GlutaMAX, and 2% Pen/Strep antibiotics). Media were replaced after 2 hr. Samples were collected at days 0, 4, 8, and 10 for RNA extraction and qRT-PCR analyses.

### 1.6. Adherent cell neuroectoderm differentiation

For the differentiation of mESCs into neural ectoderm in monolayer conditions, cells were pretreated with 10 μmol/L BPA or DMSO control as above. Then, RA was added to the medium to stimulate neuroectoderm differentiation. Samples were collected every other day from day 0 to day 10 for RNA extraction and gene expression analyses by qRT-PCR.

### 1.7. RNA extraction and qRT-PCR analyses

Total RNA was extracted with Trizol ( Life Technologies, NY, USA), following the instructions of the manufacturer. RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining. RNAs were converted to cDNAs with the PrimeScript RT Master Mix Kit (Takara, Japan) according to the manufacturer's recommended procedures. qPCR was performed with the SYBR Premix Ex Taq kit (Takara, Japan). Cycling conditions were executed forty times, except initial denaturation and final cycle, as following: initial denaturation, 95°C 30 sec; denaturation, 95°C 5 sec; annealing, 60°C 30 sec; final cycle, 95°C 5 sec, 60°C 1 min, 95°C 30 sec. The comparative $C_t$ methods was used to calculate the relative gene expression normalized to the *gapdh* gene. Measures were obtained in triplicates. Results are shown as mean ± standard deviation (SD). Primers employed are listed in Table 1.

### 1.8. Statistical analysis

Statistical significance was determined by multiple *t*-test using the Holm-Sidak method, with alpha = 5.000%. Each row was analyzed individually, without assuming a consistent SD.