Bisphenol-A Exposure Induced Neurotoxicity and Synapse and Cytoskeleton Dysfunction in Neuro-2a Cells

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Abstract

Bisphenol A (BPA) is one of the typical environmental endocrine disruptors. BPA was leached from polycarbonate containers into food and water, and it has been detected in collective samples from humans. Microtubule-associated protein 2 (MAP2) and Tau maintain microtubule normal function and promote the normal development of the nervous system. Synaptophysin (SYP) and drebrin (Dbn) proteins are involved in regulating synaptic plasticity. This study aimed to determine the adverse effects of BPA on Neuro-2a cells by investigating the synaptic and cytoskeletal damage. Cells were exposed to 0 (Minimum Essential Medium, MEM), 0.01% (v/v) DMSO and 150 µM BPA for 12, 24, or 36 h. Morphological analysis revealed that the cells in the BPA-treated groups shrank, collapsed, and had a reduced number of synapses compared with those in the control groups. CCK-8 and LDH assays showed that the mortality of Neuro-2a cells increased as the BPA treatment time was prolonged. Transmission electron microscopic analysis further revealed that cells demonstrated nucleolar swelling and nuclear membrane and partial mitochondrial dissolution or condensation following BPA exposure. BPA also significantly decreased the relative protein expression levels of MAP2, Tau, and Dbn ($P < 0.01$). Interestingly, the relative protein expression levels of SYP increased ($P < 0.01$). These results indicated that BPA damaged the development and proliferation of Neuro-2a cells by disrupting cytoskeleton and synaptic integrity.

Introduction

Environmental endocrine disruptors are a kind of exogenous chemicals, and they exist ubiquitously in the environment, affecting the endocrine system and interfering with the synthesis, secretion, and metabolism of natural hormones that affect on the reproductive system, immune system, and nervous system of humans(Frye et al. 2012). Bisphenol A (BPA) is a typical environmental endocrine disruptor used in industrial and commercial applications, such in water bottles, food and paper products, thermal receipts, storage containers, and medical products(Olea et al. 1996). Several experimental studies have shown that BPA was leached from polycarbonate containers into food and water in natural conditions and released quickly in heating, over-acid, and over-alkali conditions(Krzastek et al. 2021). BPA was detected in the urine of 95 percent of people tested in the United States; it was also detected in amniotic fluid, neonatal blood, placenta, umbilical cord blood, and human breast milk(Richter et al. 2007). A recent study indicated that the concentration of BPA was 3.2 ng/mL in serum samples collected by residents near e-waste disassembly facilities in China(Song et al. 2019). Many experiments have shown that BPA interferes with synaptic plasticity, learning and memory ability, and cognitive function and affects the development of brain and neurological behavior(Frankfurt et al. 2020; Li et al. 2021; Qin et al. 2021).

Microtubules is an important factor in controlling the transport of cellular substances and the normal function of neurons in the nervous system. When the expression of microtubule-associated proteins is abnormal, the function of the microtubule system is bound to be damaged, which induces the dysfunction of the nervous system and eventually develops into neurodegenerative diseases(Riedel et al. 2016). Microtubule-associated protein 2 (MAP2) and Tau protein (Tau) have been studied extensively, and together, they maintain microtubule function and promote the normal development of the nervous
system (Bell et al. 2021). MAP2 is a main component of the neuronal cytoskeleton, and it promotes microtubule formation, maintains microtubule stability, regulates the formation of microtubule bundles, adjusts synaptic plasticity process, and participates in neurite formation and growth, synaptic transmission in neuronal development (Murray et al. 2014). In addition, MAP2 is commonly used as a marker protein for mature neurons (Yin et al. 2020). Tau is principally enriched in neuronal axons, and it physiologically promotes the assembly and stabilization of microtubules in vesicular and axonal transport (Pérez et al. 2018). In addition, the polarity of neurons was identified by Tau (Zempel et al. 2013). Many reports have pointed out that the normal physiological function of Tau was disturbed, including genetic mutation and hyperphosphorylation that led to the occurrence of Tauopathy (Spillantini and Goedert 2013). Tau phosphorylated modification is the overwhelming ingredient of neurofibrillary tangles, which are one of the primary neuropathological hallmarks of Alzheimer disease (Cramer et al. 2018).

Synaptophysin (SYP) is the most abundant calcium-binding glycoprotein of the synaptic vesicle membrane-bound protein and the molecular marker of presynaptic density, and it is associated with synaptic plasticity and used to detect synaptic density and distribution (Zhao et al. 2017). The increased level of SYP indirectly indicated the increased release of neurotransmitters (Shang et al. 2016). In turn, the decreased level of SYP indicated a decrease in the number of synapses and synaptic plasticity and further induced the decline in learning and memory ability (Trinchese et al. 2008). Drebrin (Dbn) is an actin binding protein that exists in the dendritic spines of 70% excitatory synapses (Rao et al. 2012; Nishijima et al. 2013). Dbn is involved in regulating actin cytoskeleton structure and maintaining and adjusting the morphology and plasticity of dendritic spines (Zhou et al. 2004). The decreased level of Dbn caused collapse and atrophy of dendritic spines. The neurite length increased significantly with overexpressed Dbn, and the growth of axon was slowed down with Dbn under-expression (Hayashi and Shirao 1999).

In this study, Neuro-2a cells were selected to investigate the toxic effects of BPA on neuronal cytoskeleton and synapse. The results provide novel perspectives on the roles of SYP, Dbn, MAP2, and Tau in the potential molecular mechanisms of BPA toxicity.

Materials And Methods

Chemicals

Anti-fade reagent, bovine serum albumin, DAPI, radio-immunoprecipitation assay RIPA buffer, PVDF membranes, and goat anti-rabbit IgG (γ-chain specific) antibody were purchased from Boster, Wuhan, China. BPA (99% purity) and dimethyl sulfoxide were procured from Sigma Aldrich (St. Louis, Missouri, USA). Polyclonal rabbit anti- MAP2, polyclonal rabbit anti-Tau, polyclonal rabbit anti-Dbn, and polyclonal rabbit anti-SYP were obtained from Proteintech, Wuhan, China. Fetal bovine serum and penicillin/streptomycin were obtained from Gibco (Gibco-BRL, Gaithersburg, MD, USA). Minimum essential medium (MEM) was procured from Hyclone (Hyclone Laboratories, Beijing, China). Super ECL Chemiluminescence plus kit was obtained from Solarbio, Beijing, China. Lactate Dehydrogenase Activity
Assay Kit was procured from Nanjing Jiancheng, Nanjing, China. Skimmed milk was obtained from BD Biosciences.

**Cell cultures and treatment**

Neuro-2a cells were obtained from IBCB (the Institute of Biochemistry and Cell Biology, China). Neuro-2a cells were cultured in MEM supplemented with 10% (v/v) FBS, 50 U/mL penicillin and 50 μg/mL streptomycin. The cells were maintained at 37 °C in a humidified chamber with 95% air and 5% CO₂. When the cells were cultured to the third or fourth generation, they were used for testing. The cells were exposed to different time treatments. A stock solution of BPA was dissolved in DMSO 0.01% (v/v) after diluting to the final concentration of 150 μM of BPA-mixed MEM. The cells were seeded in 10 cm culture dishes. After 24 h, the cells were incubated with or without 150 μM BPA for 12, 24 or 36 h. The doses of BPA were selected based on the basic of our studies and previous reports (Kim et al. 2009; Tiwari et al. 2015; Cho et al. 2018; Yin et al. 2020). The DMSO 0.01% (v/v) group served as negative control. Three replicates of each cell culture were used for data analysis.

**CCK-8 and lactate dehydrogenase (LDH) assays**

Cell viability was measured using CCK-8 assay as described (Ge et al. 2019). In brief, Neuro-2a cells were seeded at a density of 2 × 10⁴ cells/well in 96-well plates and treated with 0 (MEM), DMSO, and 150 μM BPA at the designated time points. After the respective exposure, a CCK-8 reagent (5 mg/ml of stock in PBS) was added to each well and incubated for 2 h at 37 °C. The absorbance was measured at 480 nm using the Multiskan Spectrum (Thermo Fisher, USA).

Cell mortality rate was measured using the LDH assay as described (Yin et al. 2020). In brief, Neuro-2a cells were seeded at a density of 2 × 10⁴ cells/well in 12-well plates and treated with 150 μM BPA at the designated time points, or DMSO as the solvent control. After the respective exposure, the 12-well plates were shaken gently to homogenize the released LDH of the cells in the cell culture medium. The supernatants were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm and 4 °C for 15 min to discard cell debris. Then, 10 μl of LDH solution was added to the substrate solution, and the absorbance at 450 nm was measured using the Multiskan Spectrum (Thermo Fisher).

**Transmission electron microscopic (TEM) analysis**

TEM analysis was conducted to evaluate the ultra-structural changes in Neuro-2a cells after being treated with BPA at different times. In brief, the cells were seeded onto 24-well chambered cover glasses and grown under the conditions described above. After treatment, the culture supernatants were removed. The cells were fixed with 2.5% glutaraldehyde for 2 h at 4 °C, followed by 1% osmium tetroxide for 2 h at 37 °C after washing thrice with PBS. Finally, the glasses were dehydrated in a graded series of ethanol solutions and then embedded in epoxy resin. Sections (70-80 nm) were collected on copper grids and stained with 5% uranyl acetate and lead citrate. Images were captured using JEOL JEM-1230 TEM (JEOL USA, Inc.) with Gatan Ultrascan (Gatan Inc., Pleasanton, CA). The experiments were performed thrice.
**Phalloidin staining analysis**

Neuro-2a cells were seeded on coverslips in a six-well plate treated with BPA (150 μM) for 12, 24, or 36 h at 37 °C to monitor the synaptic properties of cells exposed with BPA at different times. Afterwards, the culture supernatants were removed, and the cells were fixed with 4% paraformaldehyde for 5 min after washing with PBS. The cells were then stained with phalloidin, kept away from light and incubated at room temperature for 1 h. Next, they were washed with PBS to remove uncombined phalloidin. The wells were dyed with DAPI for 5 min at 37 °C and then washed with PBS to remove the unbound DAPI. Finally, the coverslips were sealed with an anti-fade reagent and observed under a fluorescence microscope. Images were collected using LSM 800 confocal LASER scanning microscope (ZEISS, Germany) with excitation wavelengths of 488 and 561 nm.

**Protein extraction and Western blot analysis**

Total protein extractions of the samples were homogenized in a modified RIPA buffer containing 1% protease inhibitor and 1% phosphatase inhibitor for 30 min on ice-cold condition after treatment. The lysate was centrifuged at 12,000 rpm at 4 °C for 10 min, and the supernatant was obtained for protein assay. Protein concentration was determined using a Bio-Rad protein assay kit (BCA) following the manufacturer’s procedure. The 20 μg equivalent extracts of each sample were mixed with loading buffer and boiled at 95 °C for 10 min, electrophoresed on 15% SDS-PAGE, and then electro-transferred into polyvinylidene fluoride (PVDF, 0.22 μm) membranes. These membranes were blocked for 2 h with 5% skimmed milk in phosphate-buffered saline-Tween 20 (PBST). After the membranes were incubated with primary antibody solutions (MAP2-1: 1200, Tau-1: 8000, SYP-1: 2000, Dbn-1: 1500 and GAPDH-1: 1000) overnight at 4 °C, they were washed with PBST and incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. The protein blots were scanned on ECL Plus solution in ChemiDo Touch Imaging System. The intensity of the protein was analyzed using Image Lab software. The levels of SYP, Dbn, MAP2, and Tau were normalized to the level of GAPDH in each band. The experiments were repeated three times (Zheng et al. 2016).

**Statistical analysis**

Data were presented as mean ± standard error of the mean. Differences between means were determined using ANOVA for group-paired observations.

**Results**

**BPA effects on cell morphology of Neuro-2a cell**

Neuro-2a cells were treated with 0 (MEM), DMSO, and 150 μM of BPA for 12, 24, or 36 h. Morphological analysis pointed out that the Neuro-2a cells were tightly adhered to the culture vessels and appeared closely aligned in the control group (treated with MEM and DMSO, Figs. 1 A, B, D, E, G, H). The morphology of cells treated with BPA (150 μM) remained intact for 12 h (Figs. 1 C). However, the cells
treated with BPA (150 μM) shrunk, collapsed, and showed reduced number of synapses compared with the control groups for 24 and 36 h (Figs. 1 F and I). These results suggested that BPA triggered cell damage with the extension of BPA treatment time.

**BPA effects on Neuro-2a cell activity**

CCK-8 and LDH assays were conducted to further study the toxic effects of BPA on Neuro-2a cells. The results showed that the cell activity and permeability of cell membranes in the BPA group were basically consistent compared with those in the control group for 12 h (Fig. 2). Cell viability was determined with BPA after incubation for 24 and 36 h ($P < 0.01$, Fig. 2). The results of LDH assay indicated that cell mortality increased when the cells were treated with BPA (150 μM) for 24 ($P < 0.01$) and 36 h ($P > 0.05$, Fig. 2). The results illustrated that cell membranes were fragmentized with the prolongation of BPA incubation time. Thus BPA has a time-dependent manner cytotoxicity effect on Neuro-2a cells.

**TEM observation**

TEM was performed after BPA (150 μM) treatment to observe the toxic effect of BPA on Neuro-2a cells ultrastructure. The cells exhibited clear and intact nuclei, nuclear membranes, abundant mitochondria, and endoplasmic reticula in the control (treated with MEM and DMSO) and BPA groups at 12 h (Figs. 3 A, B, C, D, E, G, H). The ultrastructural changes were shown after treatment with BPA for 24 and 36 h (Figs. 3 F and I). They included chromatin sparse, intensified nucleolar swelling, partially condensed mitochondria, expanded endoplasmic reticulum, and several secondary lysosome formations. The mitochondrial damage was extensive, including vacuoles formed and fragmentized. Cellular swelling and dissolution were also observed. In particular, the nuclei exhibited irregular shapes and increased heterochromatin that were malformed, naked, and fragmentized. These results showed that the toxic effects of BPA on the ultrastructure became increasingly serious as BPA treatment time was prolonged in Neuro-2a cells.

**Results of combined phalloidin and filamentous actin (F-actin) staining**

Combined phalloidin and F-actin staining was conducted to observe the makeup of microfilament skeletons in Neuro-2a cells. The results indicated that the synapses exhibited integrity and stability in the control (treated with MEM and DMSO) and BPA groups at 12 h. The amounts of dendrites in the BPA group for 24 and 36 h decreased significantly (Fig. 4). The intensity of fluorescence was also weaker in the groups treated for 24 and 36 h than those treated for 12 h. These results indicated that BPA exerts toxic effects on the cytoskeleton of Neuro-2a cells in a time-dependent manner.

**Effects of BPA on the expression of MAP2, Tau, SYP, and Dbn in Neuro-2a cells**

The expression of MAP2, Tau, Dbn and SYP proteins were detected to further study the effect of BPA on cytoskeleton and synapsis of Neuro-2a cells. In Fig.5, the relative expression of MAP2 were decreased significantly after BPA treatment for 12 or 36 h compared with that in the control group ($P < 0.01$). The relative expression of Tau and Dbn proteins decreased significantly after BPA exposure for 12 ($P < 0.05$)
and 36 h \((P < 0.01, \text{Fig. 5})\). Interestingly, the relative expression of SYP was increased significantly following BPA exposure for 12 \((P < 0.05)\) and 36 \((P < 0.01)\) h (Fig. 5) than those in the MEM and DMSO groups. These results suggested that BPA exposure injures the function of neuronal cytoskeleton.

**Discussion**

Environmental endocrine disruptors are compounds that disturb the normal homeostasis of the endocrine system, and they cause adverse health effects with bioaccumulation in organism\(\text{(Anifandis et al. 2017)}\). BPA is one of the most typical environmental endocrine disruptors industrially produced and widely used as a component of epoxy resins and polycarbonate plastics\(\text{(Anifandis et al. 2018)}\). BPA was detected in maternal and fetal brains after subcutaneous injection into maternal rats within 1 hour. Therefore, the central nervous system is one of the best target organs to study the toxic effects of BPA\(\text{(Suzuki et al. 2002)}\).

In this study, the CCK-8 results showed that the cell activity reduced with BPA incubation for 24 and 36 h. Similar to these results, the viability reduced significantly with BPA at 200 µM treatment for 24 h in hippocampus-derived neural stem cell culture. MTT assays revealed that BPA significantly reduced neuronal viability at 200 µM but not at 50 or 100 µM\(\text{(Tiwari et al. 2015; Cho et al. 2018)}\). The LDH results showed that integrity in cell membrane permeability with BPA exposure for 12 h. The cell membrane permeability was destroyed seriously with BPA treatment for 24 or 36 h. However, remained intact with BPA exposure for 24 h in neural progenitor cells\(\text{(Kim et al. 2007)}\). This difference may be due to the different endurance capacities between the two cells or the short exposure time. In addition, morphological analysis showed that cell morphology was intact at 12 h of BPA treatment. Cells shrunk and collapsed with the extension of BPA treatment time. Phalloidin staining analysis showed that the amounts of synapses decreased at BPA exposure for 24 or 36 h. These results suggested that long-time exposure to BPA reduced survival rate, increased membrane permeability and decreased synapses. Therefore, BPA \(\text{(150 µM)}\) exhibited cytotoxicity on Neuro-2a cells in a time-dependent manner.

The stability of microtubules is an indispensable factor in maintaining the normal function of neurons in the nervous system. MAP2 and Tau participate in microtubule polymerization and synaptogenesis\(\text{(Bell et al. 2021)}\). The function of the microtubule system is bound to be damaged as the expression of the two proteins is eccentric, which induced the dysfunction of the nervous system and eventually developed into neurodegenerative diseases\(\text{(Riedel et al. 2016)}\). Neurite outgrowth and MAP2 intensity decreased at 100 µM BPA in neurons\(\text{(Cho et al. 2018)}\). BPA treatment decreased MAP2 expression at the proteomic and genomic levels\(\text{(Wang et al. 2019)}\). Other studies have reported that BPA treatment reduced the expression of total Tau\(\text{(Wang et al. 2017)}\). Similarly, previous studies indicated that BPA decreased the Tau expression levels, induced apoptosis, and inhibited neurite outgrowth in Neuro-2a cells\(\text{(Aung et al. 2013; Kumar and Thakur 2017)}\). These results are consistent with the findings in the present study, which showed that the expression levels of MAP2 and Tau were statistically decreased by BPA treatment for 24 or 36 h but not 12 h, which suggested that microtubule organization and microtubule remodeling were damaged by BPA treatment in Neuro-2a cells.
SYP is involved in the regulation of the release of Ca\textsuperscript{2+}/calmodulin-dependent neurotransmitters through phosphorylation and non-phosphorylation, and it participates in the informational transmission between neurons. SYP is a marker of synaptogenesis, and it reflects the transmission efficiency of synapse (Thiel 1993). A previous study confirmed that SYP regulated the size, number, and precocity of synaptic vesicles (Valtorta et al. 1995). Synaptic density significantly increased at 100 nM BPA for 7 days in the hypothalamic neurons of rat (Yokosuka et al. 2008). The number of spines increased with synaptogenesis (Zuo et al. 2005b, a). Spinogenesis was significantly enhanced by BPA within 2 h in hippocampal neuron cells (Tanabe et al. 2012). Nonetheless, lead exposure decreased SYP protein expression in the mouse hippocampus (Yu et al. 2016). In the present study, the expression of SYP was up-regulated with BPA treatment for 24 or 36 h. Similarly, the previous study showed that BPA exposure increased SYP protein expression at 150 and 200 µM for 24 h (Yin et al. 2020). Further studies are required to determine the reasons why BPA induced the upregulation of SYP expression and the underlying specific stimulated signaling pathways in Neuro-2a cells. Dbn is highly abundant in dendritic spines and developing neurites; it stabilizes actin filaments by sidewise binding and competing off other actin binding proteins (Grintsevich et al. 2010; Mikati et al. 2013; Kreis et al. 2019). It is also widely expressed and has mainly been studied in neurons (Aoki et al. 2005). Dbn loss leads to defective synaptic plasticity and excessive neurodegeneration following brain injuries (Schiweck et al. 2021). The results of the present study showed that the expression of Dbn decreased with prolonged treatment time. Similarly, cadmium inhibited the mRNA and protein expression levels of Dbn in Neuro-2a cells (Ge et al. 2019). These results showed that synaptic plasticity and the transmission efficiency of synapse were impaired after treatment with BPA in Neuro-2a cells.

**Conclusions**

In summary, the BPA concentration 150 µM is a safety does for 12 h in Neuro-2a cells. BPA induced synapse loss, shrunken cell body, mitochondrial swelling, and nuclear envelop breakdown at 24 or 36 h. In addition, BPA exposure reduced the expression of MAP2, Tau, and Dbn but increased that of SYP, indicating that BPA affects the synaptic plasticity and the stability of microtubules.

**Declarations**

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**Author contribution** Xinrui Wang wrote the manuscript. Zhihong Yin contributed to design and data analysis. Lingli chen: contributed to design and interpretation of results. Liushuai Hua carried out the experiments and collected data. Fei Ren, Siting Wang, Rongbo Li and Hongmei Ning contributed to revise the manuscript. Yaming Ge managed the project.

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**Availability of data** All data generated or analyzed during this study are included in this published article.

**Ethics approval and consent to participate** All of the procedures involving the cells were approved by and performed according to the guidelines of the Animal Ethics Committee of the Henan Institute of Science and Technology. All participates agreed to participate in this study and signed the informed consents.

**Consent to publication** All authors have read and approved this version of the article and consent to publish.

**Conflict of interest** All authors declare no competing interests.

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Figures
Figure 1

Effects of BPA exposure on Neuro-2a cells morphology.

Effects of BPA exposure on Neuro-2a cells morphology. A: MEM at 12 h, B: DMSO at 12 h, C: 150 μM at 12 h; D: MEM at 24 h, E: DMSO at 24 h, F: 150 μM at 24 h; G: MEM at 36 h, H: DMSO at 36 h, I: 150 μM at 36 h; a is a magnification of A; b is a magnification of B; c is a magnification of C; d is a magnification of
D; e is a magnification of E; f is a magnification of F; g is a magnification of G; h is a magnification of H; i is a magnification of I. The yellow arrow indicates the living cells. The black arrow indicates the dead cells.

Figure 2

Effects of BPA exposure on Neuro-2a cells viability.

Effects of BPA exposure on Neuro-2a cells viability and mortality. Number of surviving cells detected by the CCK-8; LDH release treated with different BPA times. *P < 0.05, **P < 0.01.

Figure 3

Transmission electron microscopic images of Neuro-2a cells exposed to BPA.

A: MEM at 12 h, B: DMSO at 12 h, C: 150 μM at 12 h; D: MEM at 24 h, E: DMSO at 24 h, F: 150 μM at 24 h; G: MEM at 36 h, H: DMSO at 36 h, I: 150 μM at 36 h; a1 and a2 are the magnification of A; b1 and b2 are the magnification of B; c1 and c2 are the magnification of C; d1 and d2 are the magnification of D; e1 and e2 are the magnification of E; f1 and f2 are the magnification of F; g1 and g2 are the magnification of G; h1 and h2 are the magnification of H; i1 and i2 are the magnification of I. Nu, nucleus; Mi, mitochondrion; NM, nuclear membrane. The yellow arrow indicates synapse.
Figure 4

Confocal microscopy and image analyses of Neuro-2a cells stained with phalloidin.

Representative images were shown to demonstrate the influence of BPA on axon spines. DAPI was used to indicate cell nucleus.
Figure 5

Effects of BPA on the expression of MAP2, Tau, SYP, and Dbn in Neuro-2a cells.

A: Optical densitometry of bands normalized to GAPDH (loading control). B: The relative protein expression of MAP2, Tau, SYP and Dbn were normalized to that of GAPDH. The bars in each panel represent the mean ± standard error of mean (SEM) (n = 3), * $P < 0.05$, ** $P < 0.01$. 