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Dose-dependent effects of Bisphenol A exposure on DNA damage in brain of young mice

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Abstract

Bisphenol A (BPA), one of the most prevalent xenoestrogens for daily use, has been reported to induce developmental neurotoxicity, but its mechanism is poorly understood. Animal studies suggest that early exposure during gestation and/or lactation to this compound may produce a broad range of adverse effects, including disruption of sexual differentiation in the brain, which could extend to future generations. Thus, it is important to investigate the extent of DNA damage induced by BPA on brain tissues during prepubertal period. Young male mice (postnatal - day 28) were exposed orally to various doses of BPA (5 µg, 50 µg, 0.5 mg, 5 mg, and 50 mg/kg bw) in drinking water for 21 days. These mice exhibited remarkable DNA damage in a dose dependent manner. Interestingly, electrophoresis of DNA from brain tissues of mice treated with high doses (5 mg and 50 mg/kg bw) demonstrated the typical ladder pattern of inter nucleosomal DNA cleavage characteristic of apoptosis. In conclusion, oral administration of different doses of BPA including environmentally relevant concentrations, had marked genotoxic effects in the brain tissues of mice during prepubertal period, and thus has set the stage for brain tumor formation later in life.

Key words : Bisphenol A; Comet assay; DNA ladders; brain; mice

INTRODUCTION

Bisphenol A (BPA), also known as 4,4'-isopropylide nediphenol, is one of the world's most widely-manufactured chemicals and can be found virtually everywhere. The ubiquitous and extensive use of BPA-containing products results in high human exposure worldwide [1], providing the potential of being exposed for neonate through ingestion of canned food, infant formula, or maternal milk. Importantly, the concentration of urine BPA was found highest in children, followed by adolescents, while the lowest levels were found in adults.[1] This has raised great concern regarding the environmental health risks to children of BPA exposure. Several recent studies have demonstrated subtle effects rather than gross macroscopic abnormalities in neonatal rodents that were exposed to BPA.[2-4] Furthermore, earlier studies have confirmed that BPA long lasting organizational effects in the brain, male and female rats.[5,6]

BPA has been listed as one of the endocrine-disrupting chemicals (EDCs) that mimic or inhibit the actions of endogenous hormones and have the potential to alter the structure and function of target organs[7]. While extensive studies have been done on the action of BPA on the endocrine system, its neurotoxic effects have not been fully explored. It has been proved that BPA has the ability to cross the blood-brain barrier (BBB) efficiently irreversibly damaging the central nervous system (CNS).[8,9] In addition, it has been reported that BPA exposure impairs hippocampal neurogenesis and spatial learning and memory[10] and disrupts neocortical development in mice.[11] These reports indicate a probability that BPA may affect the nervous system, especially brain development.The effect of BPA on the CNS may be of increased importance in fetal and postnatal brain development and now appears to be a public health concern. [12]

Previously, has been demonstrated that BPA induces many adverse effects on neonatal brain development, which is suggested to be due to oxidative stress-related cytotoxicity.[13] The brain is considered sensitive to oxidative damage because of high concentrations of unsaturated lipids, aswell as a high rate of oxidative metabolism within it's tissues. Furthermore, oxidative stress can induce many kinds of negative effects, including protein cleavage and DNA strand breakages, which could lead to cancer.[14] Furthermore, it has also previously been shown that BPA, at high concentrations, induced internucleosomal DNA fragmentation, a biochemical marker of apoptosis by a reactive oxygen species (ROS)-independent mechanism in HL-60cells.[15] Moreover, it has been demonstrated that exposure to BPA in adult male and female rats at NOAEL dose (5.0 mg) and even at 10 µg, led to significant increase in genotoxic activity in bone marrow cells and DNA fragmentation in blood lymphocyte.[16] Furthermore, recent studies have demonstrated that 2.4 µg/kg and 10 µg/kg doses of BPA are is capable of acting as a reproductive toxicant.[17,18]

Based on the reported safe and toxic doses of BPA, we aimed to investigate the extent of DNA damage in the brain tissues of mice exposed prepubertally to different ranges of doses covering the environmentally relevant exposure to BPA.

METHODOLOGY

Chemicals

Bisphenol A (CAS registry no. 80-05-7), agarose, low melting agarose, absolute ethanol 99.5%, 37% formaldehyde, glacial acetic acid, Tris-(hydroxymethyl)-amino methane (Trisbase), ethylenediaminetetracetic acid disodium salt (Na_2EDTA), Triton X-100, ethidium bromide (EtBr)) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from local commercial sources.

Animals

Young male Swiss albino mice (3 weeks old) wereobtained from the animal house of Cairo University. Mice were housed in a

temperature-controlled (24-26°C) room under a 12:12 h light/dark cycle. Animals were acclimatized for 1 week prior to BPA administration, and provided food and water ad libitum.All procedures were performed in accordance with the standards of the Institutional Animal Care and Use Committee (IACUC) at the Zoology Department, Faculty of Science, Cairo University.

Dose selection

The dosages of BPA used in this experiment were based on the no observable adverse effect level (NOAEL) and low observed adverse effect level (LOAEL) values, which are considered to be 5 and 50 mg/kg bw/day, respectively.[19] It was documented that the daily intake of BPA for infants has been documented to be 4.5 µg/kg bw/day.[20] The US Environmental Protection Agency (EPA) has calculated a reference safety dose of 50µg BPA/kg bw/day, and doses below this are considered to be low dose *in vivo*.[21, 22] Based on this information the above mentioned range of doses was selected for the current study and no toxicity tests were performed as the doses were reported to be nontoxic in an animal study.[23]

Treatment schedule

Animals were randomly caged into seven experimental groups (n=5 mice/group). The first group(I) were given only normal distilled water as the negative control group, while the mice in groups II-VII received vehicle or BPA (5 µg, 50 µg, 0.5 mg, 5 mg and 50 mg/kg bw) in drinking water for 21 days. BPA was dissolved in absolute ethanol (25 mg/ml, wt/v), and then diluted with distilled water to yield a concentration of ethanol in drinking water of 4% (vol/vol); therefore, 4% EtOH was used as the vehicle.[24] There were no mortality animals during the experimental period.

At the end of the experiment, mice were killed by cervical dislocation; the brain was removed, cleared of adhering tissues, and washed in ice-cold saline solution. Small pieces of the tissue were preserved in appropriate fixatives for histological studies and the rest of the portions stored at -20°C for further genotoxic analysis.

Estimation of DNA damage

a) Qualitative DNA fragmentation assay by agarose gel electrophoresis

DNA was isolated from the brain tissue using a salting out extraction method.[25] It was quantitated at 260 nm, electrophoresed on 1.5% agarose gel in TBE buffer, and visualized after ethidium bromide staining using GelDoc XR (BioRad Laboratories, Inc).

b) Quantitative DNA damage analysis using alkaline comet assay

For comet assay, brain tissue was processed according to the method of[26]as described by[27]for a single-cell suspension preparation. The slides were subjected to electrophoresis according to the method of[28]. Electrophoresis was conducted at 24 V (0.7 V/cm) and a current of 300 mA, using a power supply (Biorad) for 30 min at 4°C.

All these steps were performed under dimmed light, and the electrophoresis tank was covered with black paper to avoid additional DNA damage due to stray light. After electrophoresis, the slides were drained and placed horizontally in a tray. Tris buffer (0.4 M; pH7.5) was added drop wise and left for 5 min to neutralize excess alkali. Neutralizing of slides was repeated thrice. Each slide was stained with 75 µlEtBr (20 µg/ml) for 5 min, then dipped in chilled distilled water to wash off excess EtBr and a cover slip was placed over it.

Slides were placed in a dark humidified chamber to prevent drying of the gel, then they were scored within 24 h. Computerized image analysis system by use of Komet 5.0 software (Kinetic Imaging Ltd., Liverpool, UK), attached to a fluorescence microscope (Leica, Germany) equipped with appropriate filters, with simultaneous image capture and scoring of 50 cells at 400× magnification was used to measure the selected comet parameters. Images from 150 cells (50 from each replicate slide) were analyzed as per the in vivo guidelines.

The parameters selected for presentation were: (1) Percentage of DNA in tail (% tail DNA, defined as the intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as percentage), (2) Tail moment (TM, equivalent to the %DNA in the comet tail multiplied by the tail length), and (3) Olive tail moment (OTM, computed as the summation of each tail intensity integral value, multiplied by its relative distance from the center of the head, the point at which the head integral was mirrored, and divided by the total comet intensity).

Statistical Analysis

In the present study, data were statistically analyzed using Statistical Package for the Social Sciences version 20 package software (SPSS1 Statistics 20, copyright IBM Corporation 1989, 2011, USA). The mean values of DNA damage parameters (%DNA in tail, TM, and OTM) for each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test; $p<0.05$ was considered significant.Regression analyses and correlation coefficient were applied to fit the relationships between the different studied doses and the degree of DNA damage. Data were represented as mean ± standard error of mean (SEM).

RESULTS

Effect of BPA on DNA fragmentation

As shown in Fig. 1, electrophoretic separation of DNA from brain tissue exposed to different doses of BPA revealed that the ladder pattern of bands with multiple averaging was about 180 bpin length corresponding to oligonucleosomal fragments.The first sign of 180 bp could be detected in group IV (lane 4), and the intensity of the ladder bands increased with dose to reach the maximum in groups VI and VII, administered high doses of BPA, 5 and 50 mg/kg, respectively (lanes 7 & 8). No obvious DNA ladder pattern was detected in group (III) which were administered 5 µg/kg BPA (lane 3), except for light smearing appearance in locations of the DNA of high molecular size, yet no DNA fragment smaller than 1000 bp was observed.

DNA damage

Alkaline comet assay analysis indicated a gradual spreading of DNA in the developed comet tail that elevated with an increase of the administered dose of BPA in drinking water for mice (Fig.2).

The studied comet parameters (% DNA in tail, TM, and OTM) were investigated throughout the course of high and low doses of BPA. Different doses of BPA caused significant alterations for all the studied comet parameters in brain tissue (Fig. 3), in a dose dependent manner ($p < 0.05$).

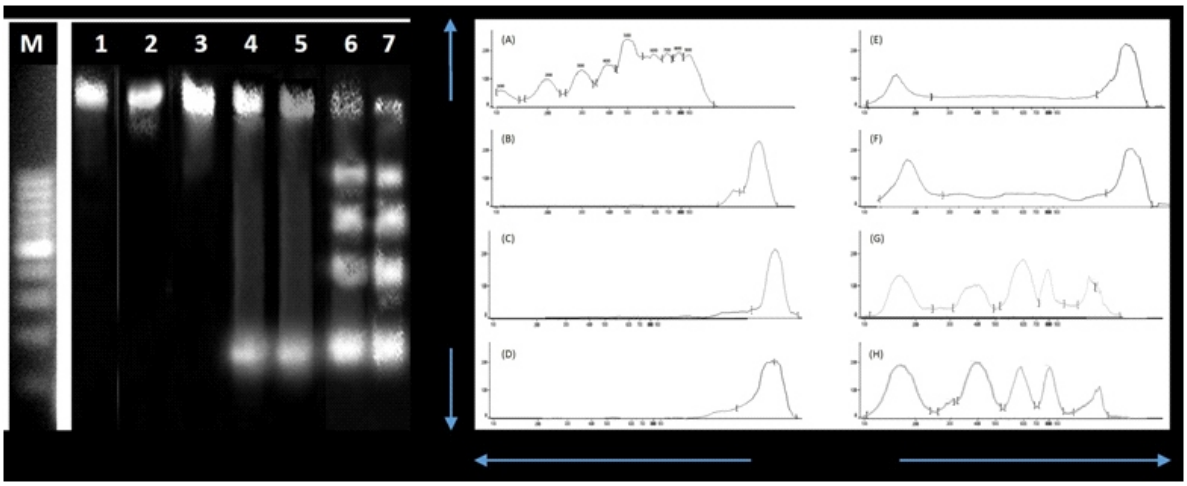

Fig 1: Induction of inter nucleosomal DNA fragmentation by BPA in mice. DNA (5-10 µg) was loaded in each lane. (Lane M):a 100 bp DNA ladder marker. (Lane 1): DNA extracted from the brain tissue of control mice. (Lane 2): DNA extracted from the brain tissue of mice given 4% ethanol (Vehicle). (Lanes 3-7): DNA extracted from the brain tissues of mice administered BPA at doses of 0.005, 0.05, 0.5, 5, and 50 mg/kg bw, respectively. Panel on right displays densitograms of corresponding lanes; the horizontal axis indicates fragment size in base pairs, while the left longitudinal axis indicates integrative optical density (IOD).

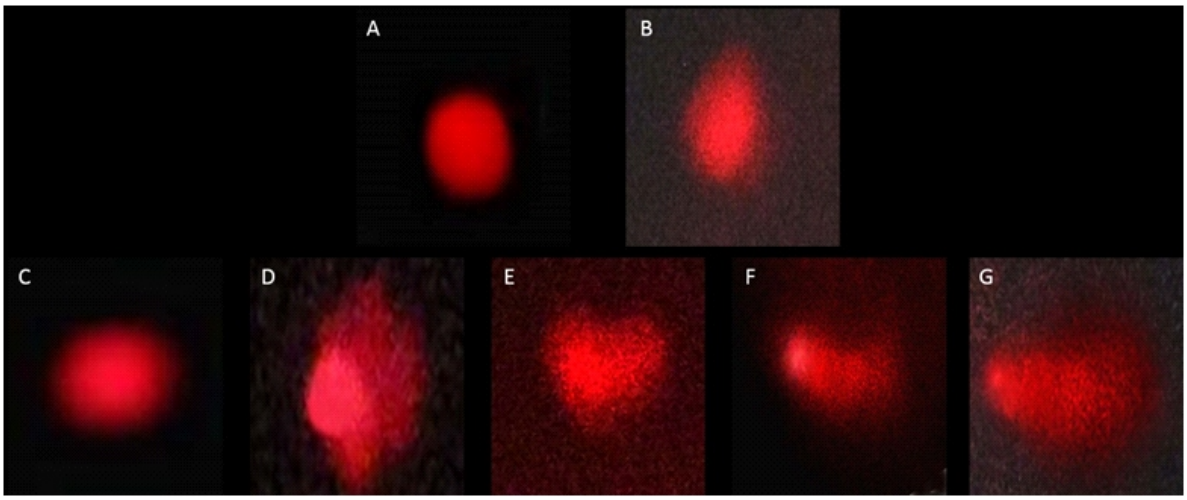

Fig 2: Extent of DNA damage assessed by comet assay in brain cells obtained from mice administered different doses of BPA. (A) Control; (B) Vehicle; (C-G) BPA treated at (C) 0.005 mg/kg; (D) 10 0.05 mg/kg; (E) 0.5 mg/kg; (F) 5 mg/kg; and (G) 50 mg/kg.

As can be seen, exposure of BPA increased DNA damage at all doses applied except in group III administered only 0.005 mg/kg. Comet assay results showed that DNA damage was significantly higher with increasing BPA doses, and an extreme elevation was observed at the highest BPA dose (50 mg/kg) administered to groups VII compared to negative control($P< 0.05$). The results revealed that the degree of DNA damage remained nearly unchanged in group III administrated the low dose of BPA (5 µg/kg) and in group II given vehicle only (Fig. 3).

Regression analysis indicated a strong positive correlation between each of the three measured comet parameters (%DNA in tail, TM and OTM) and BPA doses (Fig.4).

DISCUSSION

Bisphenol A (BPA) has been reported to have neurotoxic effects impairing learning and memory performance in young male mice[10].Although it is still unknown if BPA exerts neuronal effects on the developing brain through the action of endocrine disruption or through uncharacterized processes, it has been suggested that exposure to low doses of BPA might interfere with normal estrogenic signaling.[29] However, the precise effect and specific mechanisms have not been fully elucidated. Some reports revealed that exposure to BPA during development has persistent effects on brain structure, function and behavior in rats and mice.[30] Also, Viberg, Fredriksson, Buratovic, and Eriksson (2011)[31] declared that single exposure to BPA during the neonatal period leads to changes in brain development and in spontaneous behavior, and these functional disturbances persist, which indicates that the brain damage is permanent. Several neurotoxic endpoints *in vivo* studies suggested that BPA treatment during development could cause deleterious alterations in the brain.[32-34] Hence, there is a pressing need to investigate the underlying

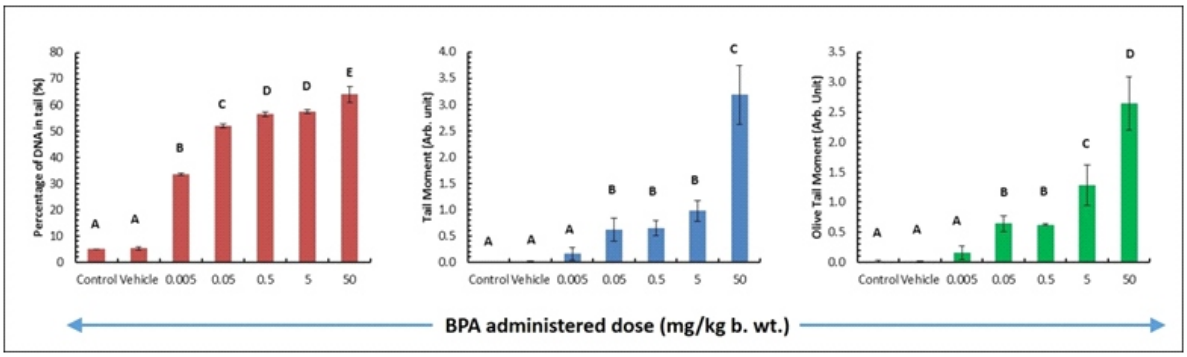

Fig 3: BPA exposure ranging from 0.005 to 50mg/kg significantly induced DNA damage, as measured by increase in percentage of tail DNA (B), tail moment (C) and Olive tail moment (D), when compared with the controls. Means with the same letter do not differ statistically at the level of $P < 0.05$.

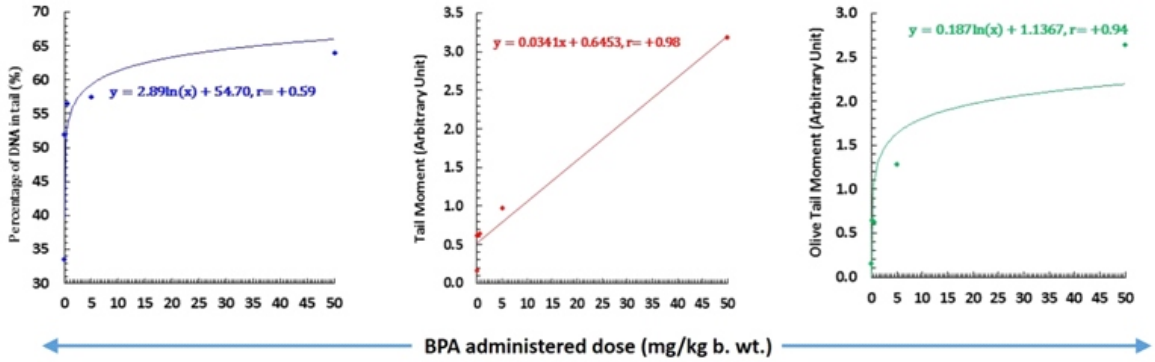

Fig 4: The relationships of graded doses of BPA (0.005, 0.05, 0.5, 5, and 50 mg/kg bw) administrated to mice in different doses with the percentage of DNA in tail (%), tail moment, and olive tail moment in the brains of mice.

mechanism of BPA neurotoxic effects on the level of DNA damage during prepubertal periods. To the best of our knowledge, this is the first study to investigatethe extent of DNA damage in the brain following exposure ofyoung mice to BPA.

Here, all treatment groups demonstrated a significant increase in percentage of tail DNA, tail moment and Olive tail moment (Fig.3 and 4)as detected by comet assay in brain tissue of young mice orally exposed to different doses of BPA. Moreover, qualitative analysis of DNA damage using agarose gel electrophoresis revealed a dose dependent manner in the degree of DNA fragmentation, with prominent apoptotic DNA laddering patterns at high doses (5 and 50 mg/kg/day). Consistent with these results, Lin, Zhang, Wang, Wu, Jiang, and Qu (2006) [35] declared that perinatal exposure of rats to BPA induces apoptosis in the midbrain when administered the same doses of BPA that exhibited apoptotic DNA laddering pattern in our study. In addition, it has been reported by Kunz, et al. (2011)[36] that direct neuronal toxicity of BPA induces neuronal apoptotic cell death, increases cell growth of immature neurons [37] and accelerates neocortical neuronal differentiation/ migration. [11]

BPA is an estrogen agonist that binds to both classical nuclear estrogen receptors (ERa and ERb), and it has been suggested that exposure to low doses of BPA might interfere with normal estrogenic signaling.[22, 38] Because BPA has been reported to affect estrogen signaling in brain tissue, it could influence estrogen signaling leading to alterations in brain functions. Studies on rats and mice have shown that early life exposure to BPA perturbs the organization of numerous estrogen-sensitive neural endpoints, including sex-specific reproductive physiology and behavior.[39] Hence, more concerns have been raised regarding the impacts of early life exposure to environmentally relevant doses of BPA on brain organizationat the cell and molecular levels. More interestingly, it was hypothesized that deregulated estrogen signaling contributes to estrogen-induced DNA damage through suppression of the DNA damage response and DNA repair pathways[40]. Therefore, we could speculate that BPA-induced DNA damage effects may occur as a result of it interfering with activity on estrogen signaling pathway. Our BPA findings are in agreement with several recent studies Iso, Futami, Iwamoto, and Furuichi (2007) [41]reported that BPA - induced DNA strand breaks in ER-positive MCF-7 cells (at 1 μmol L-1 and 100 μmol L-1) and that its genotoxicity was ER-dependent, as evidenced by much a lower effect in ER-negative cells.

BPA can penetrate the BBB and reportedly induce oxidative stress, ROS generation, and apoptosis of neuronal cells.[42, 43] It has been demonstrated that ROS may cause DNA damage, which could lead to single-strand breaks and mutation.[44] It was suggested that BPA has neurotoxic effects, which are mediated by the oxidative stress resulting from the production of free radicals.[45] This provides another probability that DNA damage observed after BPA exposure could be a consequence of free radical attack. Thus, the increase in lipid peroxidation and depletion of antioxidant enzymes in brain tissues could play a major role in BPA-induced genotoxicity.[42] Recently, BPA has been reported to enhance oxidative stress and lipid peroxidation by disturbing the redox status in cells, promoting the cellular

death in brains of exposed rodents.[42] Scientific evidence supports the hypothesis that natural estrogens, synthetic estrogen diethylstilbestrol, as well as BPA, all generate ROS during biotransformation and that certain reactive species, predominantly quinones, can react with DNA and cause DNA damage.[46]

Recently it was reported that BPA exposure in zebrafish significantly induced DNA damage associated with a significant increase of ROS production, as evaluated by alkaline comet assay.[47] They also demonstrated BPA - induced both necrotic and apoptotic cell death in whole-mount zebrafish in a dose-dependent manner, due to excess ROS-induced oxidative damage of cellular macromolecules promoting cell death. Our results revealed a significant increase in DNA damage levels observed only in the cells exposed to different concentrations of BPA. These data are in agreement with several recent studies, reporting that BPA induced DNA strand breaks and fragmentation.[15, 41, 48] In addition, BPA was reports to induce micronucleus formation and structural chromosome aberrations in bone marrow, as well as DNA damage in rats' lymphocytes.[16]

According to Xie, Nghiem, Price, and Elimelech (2012),[49] BPA has a hydrophobic nature and relatively suitable molecular dimensions and physicochemical properties to provide variable 'solute-membrane' interactions. BPA passes through the cell membrane and reaches the nucleus. It is suggested that, at relevant high concentrations, BPA binds to DNA within the nucleus, leading to destabilization as well as unwinding of the DNA, which could be a possible mechanism for its genotoxic activity. Moreover, BPA has been shown to have epigenetic effects on the expression of genes in various tissues, including in the brain and spleen[39]. In addition, BPA metabolite(s) were shown to bind to DNA in a cellular system,[50, 51] in cultured SHE cells,[52] and in rodent liver in vivo[53, 54]

CONCLUSION

In conclusion, our results report that the oral administration of BPA to young male mice for 21 days suggests that, at low doses, BPA causes oxidative DNA damage in the brain, which may be due to its estrogenic effects, while at high doses it showed genotoxic activity that may lead to apoptosis.

In the future, it will be of great interest to investigate the molecular mechanisms underlying the genotoxic or cytotoxic stress of BPA in the brain during developmental periods.

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