Original Paper

Chen et al.: Cadmium Enhances BPA Genotoxicity by OGG1 Inhibition

Cellular Physiology and Biochemistry

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Key Words
Cadmium  •  Bisphenol A  •  Genotoxicity  •  Cytotoxicity  •  OGG1

Abstract

Background: Both cadmium (Cd) and bisphenol A (BPA) are commonly encountered in humans' daily activities, but their combined genotoxic effects remain unclear. Methods: In the present study, we exposed a mouse embryonic fibroblast cell line (NIH3T3) to Cd for 24 h, followed by a 24 h BPA exposure to evaluate toxicity. The cytotoxicity was evaluated by viability with CCK-8 assay and lactate dehydrogenase (LDH) release. Reactive oxygen species (ROS) production was measured by 2',7'-dichlorofluorescein diacetate (DCFH-DA). And DNA damage was measured by 8-hydroxydeoxyguanosine (8-OHdG), phosphorylated H2AX (γH2AX) and the comet assay. The flow cytometry was used to detect cell cycle distribution, and apoptosis was determined by TUNEL assay and western blot against poly-ADP-ribose polymerase (PARP). Results: The results showed that Cd or BPA treatments alone (with the exception of BPA exposure at 50 μM) did not alter cell viability. However, pre-treatment with Cd aggravated the BPA-induced reduction in cell viability; increased BPA-induced LDH release, ROS production, DNA damage and G2 phase arrest; and elevated BPA-induced TUNEL-positive cells and the expression levels of cleaved PARP. Cd exposure concurrently decreased the expression of 8-oxoguanine-DNA glycosylase-1 (OGG1), whereas OGG1 over-expression abolished the enhancement of Cd on BPA-induced genotoxicity and cytotoxicity. Conclusion: These findings indicate that Cd exposure aggravates BPA-induced genotoxicity and cytotoxicity through OGG1 inhibition.

Introduction

It is well known that all living organisms are unavoidably and persistently exposed to a variety of synthetic chemicals through water, food, air and other sources.

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Combination exposure to cadmium (Cd) and bisphenol A (BPA) is a quite common harmful stress in both occupational and natural environments. Cd is a widespread environmental heavy metal pollutant with high toxicity that is used in smelting, battery manufacturing, pigment and plastic production, as well as in alloys, solders and electroplating [1, 2]. Thus, humans may be exposed to Cd contaminant during occupational activities. Moreover, the general population is consistently exposed to Cd through cigarette smoke and Cd-contaminated food, water, air and marine ecosystems [3, 4]. Cd compounds are known to act as carcinogens in humans and animals, targeting the lung, testis, prostate, hematopoietic cells and more [5]. Cd-induced DNA damage is considered the main mechanism of Cd-induced carcinogenesis [2]. Many previous studies have demonstrated that Cd induces DNA strand break, DNA-protein crosslinking and 8-oxoguanine formation [6, 7]. Oxidative stress is thought to be the main mechanism involved in Cd-induced genotoxicity, and it has been reported that Cd exposure increases cellular reactive oxygen species (ROS) production [8, 9]. Following their production, ROS subsequently attack cellular DNA strands or DNA bases resulting in oxidative DNA damage and cell death [6]. Moreover, antioxidant treatments protect against Cd-induced genotoxicity and cytotoxicity [10]. However, low-dose Cd exposure, which is thought to be non-toxic, enhances other factors (such as ionizing radiation, benzo[a]pyrene) that induce DNA damage and cell apoptosis [11, 12]. Investigations into these mechanisms indicate that Cd exposure inhibits the expression and activity of 8-oxoguanine-DNA glycosylase-1 (OGG1), which is a critical factor involved in oxidative DNA damage repair [13, 14]. This relationship may explain the enhanced toxicity observed when Cd exposure is combined with other toxins.

Bisphenol A (BPA), a xenoestrogenic endocrine-disrupting chemical (EDC), is widely used in the manufacturing of polycarbonate plastics and epoxy resins, such as baby bottles, dental sealants, adhesives, food packaging, and plastic beverage containers. Approximately 3.6 billion kilograms of BPA are produced and nearly 100 tons are released into the atmosphere every year. Consistent with its widespread presence, urinary BPA can be detected in more than 90% of Americans [15]. Therefore, growing concerns have emerged with regard to the possible risks of BPA to human health. Several investigations suggest that BPA exposure has been closely associated with genotoxicity and carcinogenesis [16]. The possible genotoxicity of BPA has been investigated extensively in vitro and in vivo, but the results remain controversial [17]. It has been shown that BPA can induce oxidative stress-associated DNA damage in INS-1 cells [18], micronuclei in bovine lymphocytes [19], as well as aneuploidy and DNA adduct formation [20]. However, there are also studies showing that BPA exposure fails to induce chromosomal aberrations [21] or gene mutations at the HGPRT locus [22]. Several in vivo studies also demonstrate that BPA exposure induces the formation of DNA adducts and proteome alterations in the mammary tissue of mice [23, 24]. DNA adducts were shown to form in prostate cell lines treated with either high-dose BPA for 24 h or low-dose BPA for 2 months [25]. Increased DNA damage following BPA exposure was also found in sperm DNA [26]. However, some studies indicate that BPA administration does not result in any significant induction of hyperploidy or polyploidy in sperm DNA or induce micronucleus formation in bone-marrow erythrocytes [27]. Taken together, the data remain unclear as to whether BPA exposure promotes genotoxicity in vitro and in vivo.

Based on this collective knowledge, we set out to examine whether Cd and BPA, both of which are ubiquitous in daily life, are capable of exerting a combined genotoxic and cytotoxic effect. To address this issue, we exposed a mouse embryonic fibroblast cell line (NIH3T3) to a 24 h Cd pre-treatment, followed by a 24 h BPA exposure. Following this procedure, cell cytotoxicity was assessed by CCK-8 and LDH assays and ROS production was examined. DNA damage was evaluated by detecting 8-hydroxydeoxyguanosine (8-OHdG) content, assessing γH2AX foci and protein expression and performing the alkaline comet assay. Cell cycle information was gathered using flow cytometry and cell apoptosis was determined by both the TUNEL assay and western blot against cleaved poly-ADP-ribose polymerase (PARP). Expression levels of OGG1 were examined by real-time PCR and western blot, and an OGG1
over-expression vector was constructed to characterize the role of OGG1 in the combined toxic effects of Cd and BPA.

Materials and Methods

Cell culture

A mouse embryonic fibroblast cell line (NIH3T3) was obtained from the American Tissue Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) that contained 110 mg/L sodium pyruvate, 4.5 g/L glucose, and 0.5 mM L-glutamine and was supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO₂ humidified atmosphere at 37 °C.

OGG1 over-expression

The pIRES2-EGFP vector used for OGG1 over-expression was constructed by and obtained from Invitrogen Corp.. Full-length mouse OGG1 cDNA was subcloned into the vector by DNA recombination. Cells were plated at a density of 5×10⁴ cells/ml in 6-well plates and allowed to recover and incubate for 16 h. Following this period, the OGG1 expression vector plasmid or empty vector control were dissolved into culture medium plus lipofectamine 2000 (Invitrogen Corp.) and applied to the cells for transfection. This transfection incubation continued for another 24 h. Following this period, the efficiency of transfection was evaluated by fluorescence microscopy and western blot.

Dose preparation and exposure protocols

The CdCl₂ (Sigma-Aldrich) was dissolved in distilled and deionized water to produce a 10 mM stock solution. The 100 mM stock solution of BPA (Sigma-Aldrich) was obtained by dissolving BPA powder in dimethyl sulfoxide (Sigma-Aldrich). Before application, both stock solutions were diluted in fresh cell culture medium to generate the final concentrations needed (0, 5, and 10 μM CdCl₂ and 0, 2, 10, and 50 μM BPA). Cells in the logarithmic phase of growth were pre-treated with freshly prepared 5 μM or 10 μM CdCl₂ culture medium for 24 h; then, the CdCl₂ medium was removed and medium containing 0, 2, 10, or 50 μM BPA was applied for another 24 h.

Cytotoxicity assays

Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) assays were used to examine cell viability. According to the manufacturer’s protocol, NIH3T3 cells were seeded into a 96-well plate (5×10⁴ cells/ml). Following CdCl₂ and/or BPA treatments, a mixture of 10 μL CCK-8 solution and 90 μL of culture medium were added to each well and then incubated at 37 °C for 2 h. After incubation, optical density (OD) values at 450 nm were measured with the Infinite™ M200 microplate reader (Tecan, Mannedorf, Switzerland). For each experiment, at least three replicate wells were examined. The values measured for the control group were normalized to 100%, and those values measured for the treated group were expressed as a percentage of the control.

Lactate dehydrogenase (LDH) release was used to assess the cytotoxicity induced by Cd and/or BPA. The release of LDH into the culture medium was determined using a commercially available Cytotoxicity Detection kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. In brief, following treatment, we collected cell-free culture supernatants from each well and incubated these with the LDH assay solutions for 30 min at 25 °C. The OD values at 490 nm were detected using the Infinite™ M200 Microplate Reader (Tecan). For each experiment, at least three replicate wells were examined. The values measured for the control group were normalized to 100%, and those values measured for the treated group were expressed as a percentage of the control.

Intracellular ROS assay

In accordance with previously described methods [28], ROS were measured with the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Beyotime Company, China). After cells were treated with Cd and/or BPA, their culture medium was removed, and they were washed three times with PBS.
The cells were then exposed to 10 μM DCFH-DA for 45 min, washed, and scraped from the well into 1 ml of PBS. The fluorescence was read at 495 nm for excitation and 535 nm for emission with an Infinite® M200 microplate reader (Tecan). The increase in a treated sample’s fluorescence values relative to control fluorescence values was regarded as representative of the increase in intracellular ROS.

8-hydroxydeoxyguanosine (8-OHdG) level detection

Following Cd and/or BPA treatment, DNA from each group of cells was extracted using the Genomic DNA Isolation Kit (Biovision, Milpitas, CA, USA) and quantified with Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). The level of 8-hydroxy-2-deoxyguanosine (8-OHdG) was measured using the EpiQuick 8-OHdG DNA damage quantification direct kit (Epigentek, Cambridge, UK) and detected by fluorescence at 530ex/590em nm. The results were expressed as a relative quantification (%) to a positive control provided by the kit and normalized to the input DNA (ng), according to the formula recommended by the manufacturer.

Detection of DNA damage by alkaline comet assay

DNA damage to NIH3T3 cells was assessed using an alkaline comet assay following the instructions provided by the Comet Assay Reagent kit (Trevigen, Gaithersburg, MD, USA). In brief, following Cd and/or BPA treatment, the cells were detached from the wells by trypsin digestion and, at a density of 3-5×10⁵ cells/ml, were combined with pre-warmed 0.65% low melting point (LMP) agarose at a ratio of 1:10 (v/v), placed onto Trevigen CometSlides (Trevigen) and allowed to chill at 4 °C for 30 min. Slides were then lysed in the provided lysis solution (2.5 M NaCl, 10 mM Tris HCl, 100 mM EDTA, 10% dimethyl sulfoxide and 1% Triton X-100, pH 10.0) for 2 h at 4 °C. Subsequently, the slides were placed in an alkaline buffer (200 mM NaOH, 1 mM Na₂EDTA, pH=13.0) at room temperature for 30 min. Following this incubation, the slides were subjected to electrophoresis at 1 V/cm for 40 min. The slides were then washed twice with deionized H₂O and fixed in 75% ethanol for 5 min. After air-drying, each slide was dyed with diluted SYBR Green I (Invitrogen Corp.) for 25 min at 4 °C in the dark and then examined using a fluorescence microscope (Leica, Germany). The percentage of DNA in the tail (Tail DNA%), and tail moment were selected as the main parameters used for assessing levels of DNA damage. At least 100 nucleoids of each sample were obtained in three independent experiments, and comet parameters were analyzed using a computer-based image analysis system (Comet Assay Software Project; CASP Lab, Wroclaw, Poland).

Immunofluorescence

Cells were seeded into 24-well plates with poly-L-lysine-coated coverslips at a density of 5×10⁴ cells/ml. Following treatment, cells on the coverslips were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing 3 times in PBS, the sections were permeabilized with 0.5% Triton X-100 in PBS for 30 min. Blocking was performed with 5% goat serum, followed by incubation of the slides with a rabbit polyclonal antibody against γH2AX (ab18311, 1:500 dilution, Abcam) at 4 °C overnight. The next day, after washing in PBS containing 0.5% Tween-20, the slides were incubated with an Alexa Fluor®647-conjugated goat anti-rabbit secondary antibody (1:100 dilution; Invitrogen Corp.) for 25 min at 4 °C in the dark and then examined using a fluorescence microscope (Leica, Germany). The percentage of DNA in the tail (Tail DNA%), and tail moment were selected as the main parameters used for assessing levels of DNA damage. At least 100 nucleoids of each sample were obtained in three independent experiments, and comet parameters were analyzed using a computer-based image analysis system (Comet Assay Software Project; CASP Lab, Wroclaw, Poland).

Detection of cell cycle distribution by flow cytometry

Following treatment, the NIH3T3 cells were detached by trypsin digestion, centrifuged and washed three times with chilled PBS. The cells were then fixed in 75% chilled ethanol at 4 °C for at least 24 h. Afterwards, the ethanol-fixed cells were washed twice with PBS and stained with a solution containing 50 μg/ml propidium iodide (PI) and 50 μg/ml RNase A at room temperature for 30 min with protection from light. Using these markers, cell cycle distribution was analyzed using flow cytometry and calculated using FlowJo software (BD Biosciences, USA).

TUNEL assay

Apoptotic cells were analyzed by the TUNEL assay using the In Situ Cell Death Detection Kit (Roche) following the manufacturer’s protocol. Briefly, the NIH3T3 cells were seeded on 24-well plates with poly-
L-lysine-coated coverslips at a density of 5×10⁴ cells/ml. Following treatment, the cells on coverslips were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were then permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 15 min at 4 °C. Subsequently, they were incubated with 50 μL TUNEL reaction mixture for 60 min at 37 °C in the dark. After washing, the slides were stained with DAPI for 15 min. The apoptotic (TUNEL-positive) cells were counted using a Leica confocal laser scanning microscope (TCS SP2, Germany). The apoptotic index was calculated as the ratio of the number of positive TUNEL cells over the total cells. A minimum of 500 cells was counted for each coverslip.

**Western blot**

Western blots were used for detecting γH2AX, PARP and OGG1 expression. NIH3T3 cells were seeded into 60-mm dishes at a density of 5×10⁴ cells/ml. After treatment, cells were scraped from the dish and into a lysis buffer (Beyotime Company, Jiangsu, China) containing a cocktail of phosphatase inhibitors and protease inhibitors kept on ice (Roche) and were incubated for 30 min at 4 °C. The supernatants from this lysis reaction were collected by centrifugation at 15,000×g for 30 min, and protein concentration was determined by a BCA Protein Assay kit (Beyotime). 100 μg total protein of each sample was loaded onto a 10% SDS-polyacrylamide gel for electrophoresis. The proteins were then transferred onto nitrocellulose membranes (Bio-Rad) and blocked with 5% nonfat powdered milk in PBS for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies targeting PARP (Cell Signaling Technology, Beverly, MA, USA, 1:1000), γH2AX (Abcam, 1:1000), OGG1 (Abcam, 1:1000) or β-actin (Sigma-Aldrich, 1:1000) overnight at 4 °C with gentle shaking. After washing the next day, the membranes were incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Abcam, 1:2000) for 2 h at room temperature. Then, the blots were subjected to an enhanced chemiluminescence (ECL) development system (Amersham Corp., CardiV, UK) and quantified using Quantity One image processing and analysis software (Bio-Rad).

**Real-time PCR**

According to the manufacturer’s guidelines, total RNA was extracted (Trizol, Invitrogen Corp.), RNA concentration and purity was determined by NanoDrop 2000 (Thermo Scientific), and cDNA was synthesized using a reverse transcription-PCR (RT-PCR) kit (Takara, Japan). mRNA expression of OGG1 (Forward: CAACAACATTGCTCGCATTACTG; Reverse: TCAAGCTGAATGAGTCGAGGT) was then examined using a Bio-Rad IQ5 Detection System with the SYBR Green PCR Master mix (TOYOBO, Japan). GAPDH was used as an internal control in quantitative analysis. The gene expression level was normalized to GAPDH and expressed as the percentage of the control group expression (% of control).

**Statistical analysis**

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used to perform one-way analysis of variance (ANOVA) and the Tukey test was used for the post hoc comparison of group means. At least three independent experiments were conducted. Values are expressed as the means ± standard error of mean (SEM). A p-value of <0.05 was considered statistically significant.

**Results**

**Cd pre-exposure aggravates BPA-induced cytotoxicity, ROS production and 8-OHdG formation**

Cd exposure alone at 5 or 10 μM concentration did not induce changes in cell viability, although BPA treatment alone decreased cell viability when dosed at a concentration of 50 μM. However, pre-treatment of cells with 5 μM Cd resulted in a significant reduction in cell viability in the 50 μM BPA-treated group compared to the group treated with BPA alone. Moreover, a 10 μM Cd pre-exposure reduced cell viability in both the 10 μM and 50 μM BPA-treated groups compared to those groups treated with BPA alone (Fig. 1A). LDH release assays showed that Cd exposure alone at 5 or 10 μM did not change the levels of LDH, whereas BPA treatment elevated the levels of LDH when dosed at 50 μM. Once again, pre-treatment with 5 μM Cd further increased the levels of LDH release stimulated by 50 μM BPA.
treatment, compared to the group treated with BPA alone. Furthermore, pre-exposure with 10 μM Cd significantly increased LDH release in the 10 μM BPA-treated group and in the 50 μM BPA-treated group, compared with those cells treated with BPA alone (Fig. 1 B).

Considering that oxidative stress is one of the mechanisms thought to be involved in both Cd- and BPA-induced cytotoxicity [18, 29], we examined the levels of ROS in Cd-treated, BPA-treated or combined treatment groups. The results demonstrated that Cd exposure at 5 or 10 μM did not increase levels of ROS, whereas BPA treatment alone elevated ROS production only at the 50 μM dose. However, pre-treatment of the cells with 5 μM Cd led to a significant elevation in ROS levels in both the 10 μM and 50 μM BPA-treated groups, compared to BPA treatment alone. Additionally, pre-exposure to 10 μM Cd significantly increased the levels of ROS in the 2 μM BPA-treated group, as well as those treated with 10 μM and 50 μM BPA, compared to BPA treatment alone (Fig. 1 C). It is well established that DNA oxidation results in more than 20 base modifications, the products of which (such as 8-OHdG) can be used to assess the level of oxidative DNA damage. In this study, higher levels of 8-OHdG were found in the 50 μM BPA-treated group. Interestingly, pre-treatment of cells with 5 μM Cd significantly increased the levels of 8-OHdG detected in the 10 μM and 50 μM BPA-treated groups, compared to BPA treatment alone; moreover, pre-treatment with 10 μM Cd additionally and significantly elevated the 8-OHdG levels in the 2 μM BPA-treated group, as well as in 10 μM and 50 μM BPA-treated groups compared to those treated with BPA alone (Fig. 1 D).

*Cd pre-exposure exacerbated BPA-induced DNA strand breaks*

We conducted the alkaline comet assay to evaluate the solo or combined effects of Cd and BPA exposure on cell genotoxicity. As shown in the representative images presented in
Figure 2. The effects of Cd and BPA treatment, alone or in combination, on DNA strand breaks in NIH3T3 cells. Representative images are shown from the comet assays performed in control (A), 5 μM Cd (B), 50 μM BPA (C) and 5 μM Cd plus 50 μM BPA (D) treated cells. Scale bar = 200 μm. The tail DNA% (E), and the tail moment (F) were chosen for assessing the extent of DNA damage. Representative images show γH2AX (red) foci formation in control (G), 5 μM Cd (H), 50 μM BPA (I) and 5 μM Cd plus 50 μM BPA (J) treated cells. Scale bar = 20 μm. A representative image shows γH2AX expression as detected by western blot (K). Quantitative analysis of the western blot was performed to measure γH2AX expression (L). Each value is expressed as the mean ± SEM (n=3), *P<0.01 compared to the control groups, #P<0.05 compared to the BPA alone-treated group.

Figure 2, there was no obvious DNA migration in the control or 5 μM Cd-treated groups (Fig. 2. A, B). However, there was clear DNA migration in 50 μM BPA-treated group (Fig. 2. C), and pre-treatment of cells with 5 μM Cd clearly increased the extent of DNA migration observed in the 50 μM BPA-treated group, compared to those cells treated with BPA alone (Fig. 2. D). Following quantitative analysis, neither the tail DNA% nor the tail moment were altered in the 5 or 10 μM Cd alone exposure groups. However, BPA treatment at 50 μM resulted in increased values of both tail DNA% and the tail moment. Pre-treatment of cells with 5 μM Cd, significantly increased the values of both the tail DNA% and tail moment in the 10 μM and 50 μM BPA-treated groups, compared to BPA alone. Moreover, pre-exposure to 10 μM Cd significantly increased the values of tail DNA% and tail moment in the 2 μM, 10 μM and 50 μM BPA-treated groups, compared to the group treated with BPA alone (Fig. 2. E, F).
As histone H2AX phosphorylation (also known as γH2AX) is known to be a sensitive marker for DNA double-strand breaks [30], we examined γH2AX foci formation and γH2AX expression through the use of immunofluorescence and western blot, respectively. Representative images are shown that indicate there were few γH2AX foci formed in the control or 5 μM Cd alone-treated groups (Fig. 2. G, H). However, there was an obvious increase in γH2AX foci in the 50 μM BPA-treated group (Fig. 2. I). Pre-treatment of cells with 5 μM Cd clearly increased the number of γH2AX foci in 50 μM BPA-treated group, compared to BPA alone (Fig. 2. J). Western blot results showed that there was low expression of γH2AX in the 5 and 10 μM Cd exposure groups and that BPA treatment led to increased γH2AX expression when dosed at 50 μM. However, pre-treatment of cells with 5 μM Cd significantly increased the γH2AX expression in the 10 μM and 50 μM BPA-treated groups, compared to BPA alone; and, pre-exposure with 10 μM Cd significantly increased the γH2AX expression in the 2 μM, 10 μM and 50 μM BPA groups compared to the group treated with BPA alone (Fig. 2. K, L).

**Cd pre-exposure increased BPA-induced cell cycle arrest and apoptosis**

In response to DNA damage, mammalian cells may activate a complex response network to induce cell cycle arrest and/or apoptosis. Thus, we further investigated the effects of Cd and/or BPA treatments on cell cycle distribution. Representative images indicate that there were no obvious changes in cell cycle distribution between the control and the 5 μM Cd-treated group (Fig. 3. A, B). However, there was a substantial increase in the percentage of cells in G2 phase observed in the 50 μM BPA-treated group (Fig. 3. C). Interestingly, pre-treatment with 5 μM Cd clearly increased the percentage of G2 phase cells in the 50 μM BPA group, compared to 50 μM BPA treatment alone (Fig. 3. D). Following statistical analysis, no significant differences were found in the percentages of cells in G1, S or G2 phase in the Cd alone-treated group; however, 50 μM BPA treatment did significantly decrease the percentage of cells in G1 phase and increased the percentage of cells in G2 phase but not in S phase. However, pre-treatment of the cells with 5 μM Cd also significantly decreased the percentage of cells in G1 phase and increased the percentage of cells in G2 phase in both the 10 μM and 50 μM BPA-treated groups compared to the group treated with BPA alone. Moreover, pre-exposure with 10 μM Cd significantly decreased the percentage of G1 phase cells and increased the percentage of cells in G2 phase in the 2 μM BPA-treated group, as well as in the 10 μM and 50 μM BPA-treated groups compared to the BPA alone treated group. No significant differences were found in the percentage of cells found in S phase between all groups (Fig. 3. E, F, G).

These data suggest that the treated cells triggered cell cycle arrest to provide enough time to repair damaged DNA. It is known that if DNA damage cannot be completely repaired, cells will advance to apoptosis [31]. In this study, we conducted TUNEL staining and analysis of cleaved-PARP expression levels to measure apoptosis following Cd, BPA or combined treatments. Representative images are shown that demonstrate that only a few TUNEL-positive cells were detected in both the control and 5 μM Cd-treated groups (Fig. 3. H, I). However, there was an obvious increase of TUNEL-positive cells in the 50 μM BPA-treated group (Fig. 3. J), and pre-treatment of the cells with 5 μM Cd clearly increased the number of TUNEL-positive cells in the 50 μM BPA group compared to treatment with BPA alone (Fig. 3. K). Statistical analyses did not find significant differences between the percentages of TUNEL-positive cells in the Cd alone-treated group and controls, although treatment with 50 μM BPA did significantly increase the overall percentage of TUNEL-positive cells. Once again, pre-treatment of cells with 5 μM Cd significantly increased the percentage of TUNEL-positive cells in both the 10 μM and 50 μM BPA-treated groups compared to treatment with BPA alone (Fig. 3. L). The results from the western blots demonstrated that there was low expression of cleaved-PARP in Cd exposure groups at both 5 and 10 μM and that BPA treatment increased the expression levels of cleaved-PARP only at 50 μM. However, pre-treatment of cells with 5 μM Cd significantly increased the expression levels of cleaved-PARP in the 10 μM and 50 μM BPA groups compared to BPA alone. Furthermore, pre-treatment of cells with 10 μM Cd also
significantly increased the expression levels of cleaved-PARP in the 2 μM BPA-treated group and in the 10 μM and 50 μM BPA-treated groups, compared to the group treated with BPA alone (Fig. 3. M, N).

OGG1 over-expression abolished Cd-enhanced BPA-induced genotoxicity and cytotoxicity

As OGG1 is known to play a critical role in the repair of oxidative DNA damage [32], we were interested to investigate the expression pattern of OGG1 by real-time PCR and western

Fig. 3. The effects of Cd and BPA treatment, alone or in combination, on cell cycle distribution and apoptosis in NIH3T3 cells. The representative images show the alteration to cell cycle distribution in control (A), 5 μM Cd (B), 50 μM BPA (C) and 5 μM Cd plus 50 μM BPA (D) treated cells. Quantitative analysis shows the percentages of cells found to be in G1 (E), G2 (F), and S (G) phase. The representative images show TUNEL staining (green) positive cells in control (H), 5 μM Cd (I), 50 μM BPA (J) and 5 μM Cd plus 50 μM BPA (K) treated cells. Scale bar: 50 μm. TUNEL assay results were quantitated and are shown (L). Representative image (M) and quantitative analysis (N) are shown of pro-PARP and cleaved-PARP as detected by western blot. β-actin was the internal control. Each value is expressed as the mean ± SEM (n=3), *P<0.01 compared to the control groups, #P<0.05 compared to the BPA alone-treated group.
blot in our system. We found a significant decrease of OGG1 mRNA and protein expression in Cd-treated cells at 2.5, 5 and 10 μM Cd. Following transfection with the OGG1 over-expression vector, cells were exposed to 5 or 10 μM CdCl₂ for 24 h followed by 10 μM BPA treatment for another 24 h. Following completion of both treatments, 8-OHdG content was detected (C). DNA damage was evaluated by the comet assay (D, E). Western blot for γH2AX (F) and PARP (G) was used to determine DNA double-strand breaks and apoptosis. Cell viability was detected by CCK-8 (H). A representative image (I) and quantitative analysis (J) from TUNEL assays are shown. Each value is expressed as the mean ± SEM (n=3), *P<0.01 compared to the control groups, #P<0.05 compared to the indicated group.
experiments. The results showed that OGG1 over-expression attenuated the formation of 8-OHdG in BPA-treated cells that had been pre-treated with either 5 or 10 μM Cd (Fig. 4. C). The results from comet assays showed that OGG1 over-expression abolished the increased values of tail DNA% previously observed in 5 and 10 μM Cd pre-treated, BPA-treated cells (Fig. 4. D, E). Western blots also showed that OGG1 over-expression down-regulated the increased expression of γH2AX and cleaved-PARP observed to occur in response to BPA treatment following 5 and 10 μM Cd pre-treatment (Fig. 4. F, G). Moreover, OGG1 over-expression appeared to significantly decrease the previously increased percentages of TUNEL-positive cells seen in BPA treated cells that had undergone 5 and 10 μM Cd pre-treatment (Fig. 4. I, J). Importantly, the decreased cell viability previously induced by 10 μM Cd pre-treatment, followed by BPA treatment, was blocked by OGG1 over-expression (Fig. 4. H).

Discussion

In this study, we demonstrated that treatment of cells with Cd or BPA alone (with the exception of high dose BPA exposure (50 μM) did not cause detectable changes in cell viability, LDH release, ROS production, DNA damage, cell cycle distribution or cellular apoptosis. However, pre-treatment of cells with Cd was observed to aggravate BPA-induced cytotoxicity, and increase ROS production, DNA damage, G2 phase arrest, total TUNEL positive cells and cleaved-PARP expression levels. In parallel, exposure to Cd resulted in decreased expression levels of OGG1, whereas OGG1 over-expression abolished the Cd-promoted enhancement of BPA-induced genotoxicity and cytotoxicity. Together, these findings indicate that Cd exposure sensitizes NIH3T3 cells to BPA-induced genotoxicity and cytotoxicity through OGG1 inhibition.

It have been reported that mean dissolved Cd concentrations in rivers strongly impacted by mining activities in the Riou Mort, in France is 26 μg/L [33], and the average concentrations of BPA exposure of adult is no more than 0.4-1.5 μg/kg body weight (BW) per day [34]. Although the doses of Cd and BPA used in our present study are higher than the environmental exposure levels, the aim of this study employed so high doses of Cd and BPA is to mimic the occupational exposure and to explore their combined genotoxic effects. In our study, Cd or BPA alone treatments did not show genotoxicity and cytotoxicity, with the exception of BPA exposure at 50 μM. Our results are consistent with previous data showing that BPA exposure at 25 μM does not cause oxidative stress-associated DNA damage in INS-1 cells according to the comet assay [18]. Moreover, no significant changes in cell viability or DNA damage levels have been observed in GM00637 or HeLa S3 cells when exposed to 5 μM or 10 μM Cd [14].

BPA treatment induced the increase of ROS production, which was further enhanced by Cd pre-exposure. Cellular targets for ROS are numerous and include lipids, proteins, and DNA. A major by-product of ROS attack of genomic DNA is the pre-mutagenic lesion 8-OHdG, which results in G-to-T transversions [35]. The main defense against the mutagenic effect of 8-OHdG is the base excision repair pathway, which, in eukaryotes, is initiated by the OGG1 protein, a DNA glycosylase that catalyzes the excision of 8-OHdG from DNA [36]. Improper or incomplete repairs of DNA damage due to oxidative stress may result in DNA strand breaks, cell cycle arrest, apoptosis, and mitochondrial dysfunction, which in turn increases ROS production. However, Cd is not a Fenton metal and thus does not directly increase ROS production [37]. Therefore, the enhanced ROS production induced in BPA-treated cells after Cd pre-exposure may be attributed to the inhibitory effect of Cd on OGG1 expression that we observed. In fact, it has been previously reported that over-expression of OGG1 inhibited the generation of ROS, and knockdown of OGG1 by RNA interference increased ROS generation [38].

DNA damage caused by exogenous sources, such as ionizing radiation and carcinogenic agents, are a constant threat to the genomic integrity of eukaryotic cells [39]. Genomic integrity and cell survival are closely associated with the capacity to accurately repair
environmentally or spontaneously induced DNA damage. Eukaryotic cells are equipped with a network of signal transduction pathways, namely the DNA damage response (DDR), to sense and repair damaged DNA to maintain genomic integrity [31]. When this system fails and damaged DNA is improperly repaired, cell cycle arrest and apoptosis are initiated [31, 40]. Thus, the observations of the present study that the exposure to both Cd and BPA together induced G2 phase cell cycle arrest and apoptosis suggest that these effects may be due to incompletely resolved DNA damage.

Cd exposure may down-regulate OGG1 expression and enhance cell sensitivity to oxidative DNA-damaging agents. Hartwing et al. showed that Cd is itself genotoxic and may additionally enhance the genotoxicity of other DNA damaging agents, such as UVC irradiation or alkylating agents, via inhibition of the DNA repair process [41]. For example, cadmium treatment at nontoxic concentrations increased sensitivity to UV light and enhanced UV-induced mutagenicity and cytotoxicity in V79, Hela, CHO and human fibroblast cell lines [42]. Another study also showed that treatment with cadmium at low, non-cytotoxic concentrations promoted co-genotoxic effects in combination with benzo[a]pyrene (B[a]P) [12]. Further investigation discovered that cadmium pre-treatment might sensitize cells to a dramatic increase in γ-ray-induced 8-oxoG accumulation and cytotoxicity through the suppression of OGG1 expression and subsequent DNA damage repair [14]. Many studies have revealed that Cd down-regulates OGG1 expression, which is a critical DNA repair factor involved in the excision of 8-OHdG. Cd can increase cellular apoptosis and DNA damage, decrease DNA repair capacity, and cause mutations and genomic instability, which may lead to malignant transformation. The genomic instability that results from Cd treatment may make organisms susceptible to various cancers via the inhibition of DNA repair genes such as OGG1 [43]. Through the use of OGG1 over-expression in this study, we can confirm that the enhanced genotoxicity and cytotoxicity in BPA-treated cells pre-treated with Cd can be explained by the inhibitory effect of Cd on OGG1. However, based on these data, the precise mechanism of Cd-induced OGG1 inhibition remains unknown and requires further investigation.

In conclusion, we demonstrated that Cd exposure sensitized NIH3T3 cells to BPA-induced genotoxicity and cytotoxicity through OGG1 inhibition. These findings may support the novel idea that BPA, which have been shown to be non-toxic or safe at low levels, may become highly toxic when combined with Cd. Although we could not confirm the molecular mechanisms involved in Cd-induced OGG1 inhibition, our results may provoke additional investigation into the combined effects of environmental pollutants, and future work may illuminate the underlying mechanisms of this synergistic toxicity.

Acknowledgements

This work was supported by the Outstanding Young Scientist Project of the National Natural Science Foundation of China (NO. 81422039), a Grant for Outstanding Young Scientists from Chongqing Science and Technology Commission (NO. CSTC2013jcyyjg10002) and a grant from the National Natural Science Foundation of China (Grant No: C050601).

Disclosure Statement

There are no conflicts of interest.

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