Effects of individual and combined toxicity of bisphenol A, dibutyl phthalate and cadmium on oxidative stress and genotoxicity in HepG 2 cells

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Abstract
Bisphenol A, dibutyl phthalate and cadmium can be found in environment simultaneously. Several studies suggested that they had genotoxic effect. In this study, mono-exposure and co-exposure treatments, designed by 3 × 3 full factorial, were established to determine the individual toxicity and binary mixtures’ combined effects on the oxidative stress and genotoxicity in HepG 2 cells. The highest oxidative damage was observed in the Cd treatments groups. Compared with control groups, the maximum level of reactive oxygen species and malondialdehyde were ~1.4 fold and ~2.22 fold respectively. And a minimum level of superoxide dismutase activity was found with the decrease of 43%. The mechanism that excessive oxidative stress led to the DNA damage was inferred. However, cells treated with BPA showed the worst DNA damage rather than Cd, which may because Cd mainly damages DNA repairing mechanism. For the joint effect, different interactions can be found in different biological endpoints for different combinations since different mechanisms have been clarified in mixture toxicity studies. It is sure that the co-exposure groups enhanced cytotoxicity, oxidative stress and genotoxicity compared to the mono-exposures. Synergistic and additive interactions were considered, which means greater threat to organisms when exposed to multiple estrogenic endocrine disruptors.

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1. Introduction

Three typical estrogenic endocrine disruptors (EEDs), bisphenol A (BPA), dibutyl phthalate (DBP) and cadmium (Cd), have been widely used in modern agriculture and industry. All of them are ubiquitous and persistent xenobiotic in ecosystem. BPA and DBP are widely used as important organic chemical raw materials in plastic products (Wu et al., 2011; Zhou et al., 2015). Although the half-life for bioaccumulation of them is relatively short, they still perform detrimental effects on the ecosystem as a result of their large-scale production and extensive application (Staples et al., 1998; Zhou et al., 2015). Cd, as a natural substance in crust, is an important metal material in the production of alloy, battery and insecticide etc. Due to it has a long half-life for bioaccumulation and exerts serious damage to human health, it has been classified as a toxic substance by Agency for Toxic Substances and Disease Registry (ATSDR) (Buchko et al., 2000).

It has been well documented that BPA, DBP and Cd are endogenous estrogen mimics capable contributing to endocrine disrupting effects and reproductive toxicities (Johnson et al., 2003; Mylchreest et al., 1999; Sohoni et al., 2001). According to some related studies, other types of toxic effects can be also found, such as oxidative stress and genotoxicity. BPA is able to induce oxidative stress in the liver of rats by decreasing superoxide dismutase (SOD), catalase, glutathione reductase and glutathione peroxidase activity (Bindhumol et al., 2003). Several studies demonstrated that BPA could produce cytogenetic and genotoxic effects in vitro assay in different degrees (Parry et al., 2002; Tsutsui et al., 1998; Xin et al., 2015). However, there are some controversial results indicating that BPA failed to induce gene mutations in both SHE and V79 cells.
between theoretical values and measured values at p<0.05, p<0.01, respectively; a, b in co-exposures represent difference between theoretical values and measured values at p<0.05, p<0.01, respectively.

(Pacchierotti et al., 2008; Schweik et al., 1998). Previous study suggested DBP exposure not only lead to oxidative stress but also affect lipid, energy and osmoregulation metabolism disturbance in abalone (Zhou et al., 2015). Zhou et al. (2010) also showed that DBP may alter the structure and function of the epididymis of adult rats by inducing oxidative stress. In addition, DBP and its isomer di-iso-butyl-phthalate (DiBP) exhibited genotoxic effect in human epithelial, mucosal and lymphocytes cells (Kleinsasser et al., 2000, 2001). Several reports proved that Cd led to apoptosis both in various tissues and cells (Halbeau et al., 1998; Szuster et al., 2000; Wätjen and Beyersmann, 2004; Xu et al., 1996). It appears that the apoptotic effects result from Cd are partly mediated via induction of oxidative stress (Wätjen and Beyersmann, 2004). Moreover, one possible genotoxic mechanism of Cd is oxidative stress and inactivation of several DNA-repair enzymes (Waisberg et al., 2003).

The natural co-occurrence of BPA, DBP and Cd is obvious, especially in aquatic ecosystems. It has been widely reported that the water was polluted by the three substances simultaneously in the main river in China such as the Yangtze River (Shi et al., 2011; Zhang et al., 2012), the Yellow River (Bai et al., 2012; Hongjun et al., 2013; Wang et al., 2012) and the Pearl River (Geng et al., 2015; Li et al., 2016; Wei et al., 2011). As a consequence, human population may be exposed to multiple EEDs through several ways e.g. the bathing routes, consuming contaminated water and aquatic organisms etc. Multi-exposure may lead to additive, synergistic or antagonistic effects. According to previous studies, the potential combined toxicity of EEDs should not be ignored (Kortenkamp, 2007). However, few studies on the possible EEDs interactions have been reported. Thus, it is important to evaluate the combined toxicity of BPA, DBP and Cd.

Factorial designs, one approach to determine the existed interactive effects between two (or more) mixed chemicals, in which Xₙ (n represent the number of chemicals in the mixture; x means dose levels) treatment groups is studied. This is a valuable statistical method, verified by the US Environmental Protection Agency for taking full advantage of the obtained data, is applied widely because it does not need to calculate some toxicity indexes such as EC₅₀, LD₅₀ etc. (Gronen et al., 1996). HepG2 cells are a commonly used human-derived hepatoma cell line that maintained some of phase II metabolizing enzyme activity in the liver (Otto et al., 2008; Knasmüller et al., 1998). However, the content of cytochrome P450 enzymes is very limited and the P450-mediated drug metabolism of compounds cannot be expected to take place in HepG2 cells (Rodriguezantona et al., 2002). While the 5th International Work-shop on Genotoxicity Testing (IWGT) reported that human p53-competent cells, such as HepG 2, may be a good model for in vitro mutagenicity assays in mammalian cells – in vitro micronucleus or aberration tests – in order to reduce non-relevant positive results (Corcuera et al., 2011; Pfuhler et al., 2011). So we selected factorial designs as evaluation method and HepG2 cell line as subject for research.

The objective of this study was to assess the individual and combined toxicity of BPA, DBP and Cd on oxidative stress and genotoxicity in HepG2 cells. A 3 × 3 full factorial design was used to evaluate the interactive effects of binary mixture. Three oxidative stress biomarkers (ROS: Reactive Oxygen Species; MDA: Malondialdehyde; SOD: Superoxide Dismutase) were selected to conduct in vitro mutagenicity assays in mammalian cells (Keränen et al., 2011). The HepG2 cell line is an immortalized human hepatoma cell line that maintained some of the properties of the human liver (Otto et al., 2008; Groten et al., 1996). HepG 2 cells are a commonly used human-hepatoma cell line that maintained some of the properties of the human liver (Otto et al., 2008; Groten et al., 1996). HepG 2 cells are a commonly used human-derived hepatoma cell line that maintained some of the properties of the human liver (Otto et al., 2008; Groten et al., 1996).

### Table 1

Cytotoxic effect on HepG 2 cells after 24 h, resulting from in vitro exposition in BPA, DBP and Cd alone or in combination.

<table>
<thead>
<tr>
<th>BPA (mol/L)</th>
<th>Measured value</th>
<th>Theoretical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁸</td>
<td>1.13 ± 0.06*</td>
<td>1.80 ± 0.05</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.19 ± 0.05*</td>
<td>1.40 ± 0.07**</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>1.28 ± 0.06*</td>
<td>1.50 ± 0.04**</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>1.20 ± 0.04**</td>
<td>1.38 ± 0.04**</td>
</tr>
<tr>
<td>2 mM H₂O₂</td>
<td>1.80 ± 0.05**</td>
<td>2 mM H₂O₂</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD of three replicates. * indicates difference between control and treatments groups at p<0.05, p<0.01, respectively.

### Table 2

Effect of BPA, DBP and Cd alone on intracellular ROS generation in HepG-2 cells after 6 h incubation.

<table>
<thead>
<tr>
<th>BPA (mol/L)</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.13 ± 0.06*</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>1.11 ± 0.03*</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.17 ± 0.08*</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>1.29 ± 0.07**</td>
</tr>
<tr>
<td>2 mM H₂O₂</td>
<td>1.38 ± 0.04**</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD of three replicates. * indicates difference between control and treatments groups at p<0.05, p<0.01, respectively.
dimethylsulfoxide (DMSO) to prepare the stock solutions (1 mol/L for BPA and DBP) for the further toxicity evaluations. DMSO was used as the solvent control, and the concentration was 0.1%. 1 mol/L Cadmium chloride (CdCl₂) stock solution was prepared with phosphate-buffered saline (PBS, Hyclone SH30256.01B). All stock solutions were filtered with 0.22 μm membrane and then diluted with cell culture media.

2.2. Cell culture

HepG2 cells were obtained from biochemistry laboratory (first affiliated hospital of Jinan University, Guangzhou, China). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Hyclone SH30022) which contained 10% fetal calf serum (Hyclone SH30070.03) and 1% penicillin/streptomycin solution (Hyclone SV30010) in an incubator with a normal condition (5% CO₂ and 95% air, 37 °C and 90% of humidity).

2.3. Experimental design

HepG2 cells were used to assess the oxidative stress and genotoxicity. Thereafter, the cells were exposed to BPA at following concentrations: 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/L; DBP at 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/L and CdCl₂ at 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/L. The selected concentrations were referred to previous literature (Kleinsasser et al., 2001; Wätjen and Beyersmann, 2004; Xin et al., 2015) and to ensure above 50% cell viability which was essential for comet assay (Garaj-Vrhovac et al., 2013). To determine the combined toxicity of the binary mixtures, the cells were exposed to the mixtures of two substances by a full 3² factorial design.

2.4. Cytotoxicity assays

The methyltetrazolium (MTT) method was used to determine cell viability based on reference with slightly modified (Nwagbara et al., 2007). HepG2 cells were seeded on 96-well plates (10,000 cells per well) for 24 h. Then the culture medium was removed and the cells were exposed to 100 μL fresh medium containing the selected concentrations of BPA, DBP and Cd. All samples were tested in triplicate. Solvent control (DMSO) was also assayed. After a 24 h treatment, add 10 μL MTT (5 mg/ml) to each well and then the plates was incubated for 4 h, after that remove the medium and add 100 μL DMSO. Absorbance was acquired at 490 nm with an microplate reader (SYNERGY H1). Cell viability compared solvent control was calculated from the mean absorbance values of three replicates.

2.5. Oxidative stress biomarkers response assays

HepG2 cells were cultured in 6-well plates (1.5 x 10⁵ cells/well)

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Effect of BPA, DBP and Cd alone on MDA content in HepG-2 cells after 6 h exposure.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA (mol/L)</td>
<td>MDA content (nmol/mgprot)</td>
</tr>
<tr>
<td>0</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>0.38 ± 0.007**</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>0.40 ± 0.015**</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>0.44 ± 0.026**</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>0.50 ± 0.034**</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0.55 ± 0.037**</td>
</tr>
<tr>
<td>2 mM H₂O₂</td>
<td>0.75 ± 0.018**</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD of three replicates. *, ** represent difference between control group and treatments groups at p < 0.05, p < 0.01, respectively.
for 24 h. Then the cells were treated with the selected concentrations of BPA, DBP and Cd for 6 h. Solvent control (DMSO) and positive control (2 mM H₂O₂ for last 30 min) were conducted in all tests. After treatment, the HepG2 cells were rinsed with PBS and then trypsinized to detach followed by suspended in complete medium. Cells were centrifuged and re-suspended (1 × 10⁶ cells/ml) in PBS.

Intracellular ROS were assayed using 2.7-dichlorofluorescein diacetate (DCFH-DA) as a probe. The principle of the method is that ROS can oxidize DCFH to fluorescent dichlorofluorescein (DCF). After 6 h exposure, cells were collected and washed twice with DMEM. Then 200 μL DCFH-DA (10 μM) were added and cells were incubated at 37 °C for 20 min in dark. Subsequently, cells were rinsed with DMEM. The mean fluorescent intensities were obtained by a flow cytometry (Gallios, USA). The relative fluorescence intensity (%) compared with solvent control was determined.

The collected cells were broken by ultrasonic processor and the cellular fragment was used to determine the content of MDA the vitality of SOD. The commercially available kits provided by Nanjing Jiancheng Biology Engineering Institute were used for MDA and SOD assays according to the manufacturer’s instructions.

2.6. Comet assay

HepG2 cells were seeded and collected as above, but the exposure time was 24 h. Solvent control (DMSO) and positive control (200 μM H₂O₂ for last 30 min) were conducted in all tests. DNA damage was evaluated by alkaline single cell gel electrophoresis (SCGE) method according to study with slightly modified (Collins, 2004). Briefly, 10 μL of cell suspensions were added to 75 μL 0.7% low melting point agarose (LMA). The mixture was spread onto microscope slides which were pre-coated with 0.5% normal melting point agarose (NMA). The agarose was solidified at 4 °C for 20 min. Then slides were lysed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10% DMSO and 1% TritonX-100) at 4 °C for 1 h. Slides were immersed in electrophoretic buffer (1 mM Na₂-EDTA, 300 mM NaOH, pH > 13) for 40 min to unwind DNA before electrophoresis. The electrophoresis was then carried out at 25 V for 25 min followed by neutralizing in 0.4 M Tris-HCl buffer solution (pH 7.5) three times for 10 min. Subsequently, the slides were stained with 20 μL propidium iodide (PI) and observed with a fluorescence microscope (Olympus IX 53). The most reliable parameters, tail DNA percentage (%) were calculated by comet assay software project (CASP) to evaluate DNA damage.

2.7. Statistical analysis

All experimental data indicates as mean values ± standard deviations of the replicated experiments. For individual toxicity, the statistical significance of parameters in cytotoxicity assays, oxidative stress biomarkers response assays and comet assay between control and the tested samples were analyzed by SPSS 19.0 for Windows with one-way analysis of variance (ANOVA). P < 0.05 was considered statistical significance. For the combined toxicity, three types of combined effects, i.e. additive, synergism and antagonism effect, can be observed when more than one kind of chemicals are simultaneously applied to a same system (Groten, 2000). For the binary mixtures, the interactive effects was analyzed by compared the “measured values” defined as examined endpoints to the “theoretical values” defined as sum of toxic effect values induced by each chemical alone in the mono-exposure experiments (Klaric’ et al., 2012; Klaric’ et al., 2010; Weber et al., 2005). In order to facilitate understanding, the theoretical values (T) expressed as following formula. When the results of treatment groups were...
below control groups, such as cell viability and superoxide dismutase activity, the formula was as follows:

\[ T = C_{\text{mix}} - (C_a - M_a) - (C_b - M_b) \]

When the results of treatment groups were above control groups, such as reactive oxygen species level, malondialdehyde content and tail DNA percentage (%), the theoretical values was as follows:

\[ T = C_{\text{mix}} + (M_a - C_a) - (M_b - C_b) \]

\( C_{\text{mix}} \) represented control groups' result in the co-exposure treatments. \( C_a, C_b \) represented control groups' results in the a/b mono-exposure treatments, respectively. \( M_a, M_b \) represented measured results in the a/b mono-exposure treatments, respectively.

The significance of difference between measured and theoretical values was determined with one-way ANOVA to assess if any interaction existed in. If the values of \( p \) were less than 0.05, the results implied to be significant and interaction effect i.e. synergic or antagonist effect existed whereas an additive effect was considered to be.

3. Results

3.1. Cell viability for HepG 2 cells exposed to BPA, DBP, or Cd alone and their binary mixtures

The viability of HepG 2 cells, a 24 h exposure to single and binary mixture, was measured by MTT assay and presented in Table 1. The results were expressed as a percentage compared with corresponding solvent control. Exposure to BPA and Cd alone resulted in a dose-dependent increase of mortality and statistical significance \( (p < 0.05) \) while DBP showed the similar result at \( 10^{-6} \text{ mol/L} \). Compared the cytotoxicity under the same concentration of the three \( \left( 10^{-6}\text{ mol/L} \right) \), Cd was the most effective in decreasing cell viability and BPA was less than Cd at the level above \( 10^{-6} \text{ mol/L} \).

Contrast with BPA, DBP or Cd alone \( 10^{-8} \text{ mol/L} \), a stronger cytotoxicity was found after an exposure to binary mixture. Generally, when the level of BPA in BPA + DBP or Cd in DBP + Cd increased, the cell viability was reduced more serious and the statistically significant difference existed. The combination of BPA + DBP \( \left( 10^{-6} + 10^{-6} \text{ mol/L} \right) \) showed the strongest cytotoxicity as the greatest difference between the theoretical values \( (67.2 \pm 2.6\%) \) and the measured values \( (54.0 \pm 1.2\%) \). Moreover, the combination of BPA + Cd presented almost no significant difference when compared with the theoretical values.

3.2. Effects of single or combined groups on induction of intracellular reactive oxygen species (ROS)

The level of intracellular ROS was researched after HepG 2 cells were exposed to single chemical (Table 2) or their binary mixture (Fig. 1) for 6 h. For the single exposure, almost all experiment groups were significantly increased. DBP treated cells presented a lower ROS level and a dose-dependent increased. BPA or Cd treated cells both suffered a stronger oxidative damage as ROS production increased ~1.4 fold and performed a similar tendency that both declined at the high concentration level (BPA at \( 10^{-3} \text{ mol/L} \) and Cd at \( 10^{-6} \text{ mol/L} \)) and then increased at the higher level. Combined treatments generate greater amounts of ROS than that of single exposure. Compared with the theoretical values, the measured values of the binary mixtures that Cd was contained (Fig. 1B and C) were statistically significant. However, the BPA + DBP treatments (Fig. 1A) showed no significant difference in statistics.

3.3. Lipid peroxidation (LPO) level and antioxidant activity in cells exposed to single or combined groups

Table 3 and Fig. 2 demonstrated the LPO induced by individual and joint treatments respectively. The activity of superoxide dismutase (SOD) exhibiting cellular antioxidant capacity was described in Table 4 and Fig. 3. The results showed significantly increased MDA level and decreased activity of SOD in cells treated with BPA, DBP or Cd alone compared with the solvent control. Cd treated groups showed the most serious oxidative damage as a maximum MDA level increased 221% and SOD activity reduced 43%.

The co-exposure treatments had higher MDA levels and lower SOD activity than the mono-exposure treatments. Compared with the theoretical values, the measured MDA content remarkably increased while the variation trends of SOD activity of combined groups were different. The measured SOD activity of BPA + DBP \( \left( 10^{-8} \text{ mol/L} \right) \) treatments (Fig. 3A) was almost equal to theoretical values while at \( 10^{-6} \text{ mol/L} \) BPA + \( 10^{-6} \text{ mol/L} \) DBP significantly decreased. The variation of BPA + Cd (Fig. 3B) and DBP + Cd (Fig. 3C) treated groups was similar. All of the measured values were lower than theoretical values.

3.4. Effects of BPA, DBP, or Cd alone and their binary mixtures on DNA strand breaks (comet assay)

Results of comet assay for mono-exposure and co-exposure were exhibited in Fig. 4. Compared with negative control, all HepG 2 cells exposed individual groups (Fig. 4A) resulted in a significant dose-response increase. The genotoxicity of DBP was the weakest while BPA was the strongest due to the %DNA in tail \( (21.0 \pm 2.21 - 27.6 \pm 2.37) \). BPA was almost two times higher than that treated with DBP \( (11.5 \pm 1.15 - 17.7 \pm 2.41) \). At the lowest concentration \( (10^{-8} \text{ mol/L} \) ), %DNA in tail of Cd \( (11.0 \pm 1.95) \) was nearly equal to DBP’s \( (11.5 \pm 1.15) \). However, the genotoxicity of Cd was significantly greater than DBP’s with the concentration increasing.

The combined treatments had stronger DNA damage effect (Fig. 4B). The theoretical values were shown in Table 5. The
measured genotoxicity of BPA + DBP were remarkable greater than the theoretical. For the mixtures of BPA + Cd (10⁻⁸ mol/L) and DBP + Cd (10⁻⁸ mol/L), the values of % DNA in tail had slightly differences even lower than the theoretical, moreover, as the concentration of Cd raised to 10⁻⁶ mol/L, the significantly differences, i.e. enhanced genotoxicity, could be found between the measured values and the theoretical values.

4. Discussion

BPA, DBP and Cd applied widely in consumers (Kortenkamp, 2007). Until now, several studies regarding the toxicity of the BPA, DBP or Cd have been conducted to shed some light on potential threat in humans, animals and cell, but almost all of them have been performed under mono-exposure condition. However, the combined toxicity effect also should be paid more attentions because it is a higher probability that organisms are exposed to several EEDs simultaneously. The present study demonstrated that not only BPA, DBP and Cd alone could increase the oxidant damage and DNA damage but the binary mixtures have stronger degree of damage, which means that additive or synergic effect exists.

The cytotoxicity is a basic parameter to evaluate the toxic effect of pollutants. In single 24 h treatments, it could be observed that the order of cytotoxicity was Cd > BPA > DBP. This may be a reason that Cd could induce the highest reactive oxygen species (ROS) level and reduce the superoxide dismutase (SOD) activity to a most extent. Wätjen and Beyersmann (2004) have proved that Cd induced apoptosis mainly by oxidative stress. In 24 h co-exposures, the shown cytotoxicity was more serious than mono-exposures. The results of all the BPA + DBP and DBP + Cd treatments were concordant with a synergic effect since the fact that the remaining cell viability after combined groups had significant difference with theoretical surviving cells. However, the BPA + Cd was almost an additive effect, which indicated that either the EEDs possessed the same cell pathways on cytotoxicity, or they may share some steps in toxic action. In a word, they had additive effects at least (Speijers and Speijers, 2004).

In the present study, vary degrees of DNA damage (Fig. 4) was observed in HepG 2 cells after different exposure ways. To detect the possible mechanisms of DNA damage and cell death, ROS, MDA and SOD were determined. All of the three EEDs could rise oxidative stress due to ROS level and MDA content significantly increased but SOD activity reduced. Each substance was neither nanoparticle soluble compounds that can generate ROS directly (Wilson et al., 2007) nor the substance which can generate ROS by Fenton reactions (Birmili et al., 2006). The mechanism of oxidative damage may be mainly by inhibiting antioxidant enzyme system activity. When the damage is so severe that exceed the cellular intrinsic repair capacity, DNA damage or cells death will occur.

Synergetic interaction of all the BPA + DBP treated doses were observed in MDA content while additive effect was found in ROS level and SOD activity. For the co-exposure of BPA + Cd (ROS and SOD: all the treated doses; MDA: 10⁻⁸+10⁻⁶, 10⁻⁶+10⁻⁸ and 10⁻⁶+10⁻⁸ mol/L) and DBP + Cd (ROS and MDA: 10⁻⁸+10⁻⁶, 10⁻⁶+10⁻⁸ and 10⁻⁵+10⁻⁸ mol/L; SOD: all the treated doses), the combined effect of all the three oxidative stress endpoints was almost synergetic. Different interactions, biochemical or physical interaction, in different biological markers can be observed since different mechanisms can be found in mixture toxicity studies (Wu et al., 2012). The excessive ROS could reduce antioxidant activities of SOD, which may be the possible reason why the combined effect
of ROS and SOD were same (Yu et al., 2015). Either BPA or DBP might generate oxidative damage through the similar way in their mixtures, while their potencies were different. Besides, both BPA + Cd and DBP + Cd treatments notably enhanced oxidative stress, the possible reason was that Cd could inhibited strongly detoxification process of ROS thus damage was aggravated (Wätjen and Beyersmann, 2004).

The modified Comet assay (Singh et al., 1988) which was a useful tool to evaluate environmental genotoxicity of environmental pollutants (Papis et al., 2011) was used in this work. Obviously, the present study confirmed that BPA, DBP or Cd could cause severe DNA damage, and the order of genotoxicity was BPA > Cd > DBP. Synergic effect was observed for the combination of BPA + DBP on HepG 2 cells. Strangely, the interaction between BPA/DBP and Cd was additive even antagonism at lower dosages of Cd. A possible explanation was that Cd could modify the CYP1A1 activity thus the DNA damage may be induced by BPA/DBP metabolites which was weaker genotoxicity (Vincent-Hubert et al., 2011). Moreover, at higher dosages of Cd, enhanced DNA damage can be found and the combined effect was Synergic effect in the BPA/DBP + Cd

![Representative images of comets which represent different classes of genotoxic effects of HepG 2 cells after 24 h (A) mono-exposures and (B) co-exposures. The numbers mean tail DNA percentage (%).*, ** in mono-exposures represent difference between control group and treatments groups, while in co-exposures represent difference between theoretical values and measured values at p < 0.05, p < 0.01, p < 0.001, respectively.](image)

### Table 5
The theoretical values of %DNA in tail in the co-exposure treatments.

<table>
<thead>
<tr>
<th>%DNA in tail</th>
<th>BPA and DBP (mol/L)</th>
<th>BPA and Cd (mol/L)</th>
<th>DBP and Cd (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical value</td>
<td>10⁻⁸⁻10⁻⁴</td>
<td>10⁻⁸⁻10⁻⁴</td>
<td>10⁻⁶⁻10⁻³</td>
</tr>
<tr>
<td>%DNA in tail</td>
<td>29.2 ± 1.68</td>
<td>35.4 ± 2.31</td>
<td>35.8 ± 1.76</td>
</tr>
<tr>
<td>Theoretical value</td>
<td>10⁻⁸⁻10⁻⁴</td>
<td>10⁻⁸⁻10⁻⁴</td>
<td>10⁻⁶⁻10⁻³</td>
</tr>
<tr>
<td>%DNA in tail</td>
<td>19.2 ± 1.56</td>
<td>32.0 ± 1.09</td>
<td>25.4 ± 2.19</td>
</tr>
<tr>
<td>Theoretical value</td>
<td>10⁻⁸⁻10⁻⁴</td>
<td>10⁻⁸⁻10⁻⁴</td>
<td>10⁻⁶⁻10⁻³</td>
</tr>
<tr>
<td>%DNA in tail</td>
<td>28.7 ± 2.09</td>
<td>41.5 ± 2.12</td>
<td>35.3 ± 2.17</td>
</tr>
</tbody>
</table>
treatments. The possible reason was that Cd affected the stability of the DNA double-strand not only by increasing oxidative damage but also by inhibiting several DNA-repair pathways (Bertino and Averbeck, 2006). Taken together, these results suggested that the genotoxicity on HepG 2 cells of all the binary mixtures and single substances can’t be ignored. Although several regulatory bodies, such as European Aviation Safety Agency, have concluded that single BPA does not pose a genotoxic risk to humans, the multi-exposures including BPA should be paid more attention due to the toxicity may be enhanced remarkably through synergistic and additive interactions. So people exposed in the environment of EEDs’ mixtures may bear greater health risks and the regulatory authorities should attach importance to EEDs pollution.

5. Conclusion

In this laboratory study, BPA, DBP and Cd could induce cell death, oxidative damage and DNA damage in HepG 2 cells. Cd treatment groups showed the strongest cytotoxicity and oxidative stress especially for the decreasing superoxide dismutase (SOD) activity. The strongest genotoxicity for mono-exposure was observed in BPA treatments by comet assay. The possible mechanism was that excessive oxidative stress led to the DNA damage as well as cell death. As for joint effect of binary mixtures, enhanced toxicity was demonstrated compared with BPA/DBP/Cd alone, different interactions can be found for different endpoints but synergistic and additive interactions were the mainly combined effect. It suggested that the organisms exposed to multiple estrogenic endocrine disruptors (EEDs) may have more risks than expected. Our work provides information on combined toxicity investigations on EEDs, and further researches are needed to improve and perfect risk assessment.

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