Individual and combined developmental toxicity assessment of bisphenol A and genistein using the embryonic stem cell test in vitro

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Abstract

The potential developmental toxicity of environmental estrogenic endocrine disruptors have become a great concern in recent years. In this study, two typical environmental oestrogens, namely, bisphenol A (BPA) and genistein (GEN) were investigated for potential embryotoxicity using the embryonic stem cell test model. Afterwards, a 4 × 4 full factorial design and the estimated marginal means plot were performed to assess the combined effects of these two compounds.

According to the linear discriminant functions and classification criteria, bisphenol A and genistein were classified as weakly embryotoxic and strongly embryotoxic respectively. As for combined effects, the overall interaction between BPA and GEN on embryonic stem cells (ESCs) differentiation was synergistic at low dosages, however, on ESCs and 3T3 cell proliferation, the predominate action was additive. Considering the actual daily intake of these chemicals, it is concluded that BPA alone might not have adverse reproductive or developmental effects on human being. However, given that BPA and GEN do have synergistic effect at low concentration, they may disturb normal embryo development together, which could result in birth defect and behavioral alterations later in life.

1. Introduction

Bisphenol A (BPA), a typical estrogenic endocrine disruptor (EED), has been used worldwide in the production of polycarbonate plastics, epoxy resins, and dental sealants. According to a related study (Vandenberg et al., 2007), human exposure to bisphenol A comes mainly from the daily diet, as BPA can leach from food and beverage containers as well as from dental sealants. Leaching is particularly strong in heat and either acidic or basic conditions. Thus, widespread and continuous exposure leads to detectable levels of BPA in the urine, amniotic fluid, maternal and fetal plasma, and the placental tissue of pregnant women, as well as in breast milk of lactating mothers, which has all been shown in the cross-sectional National Health and Nutrition Examination Survey study (NTP-CERHR, 2008). Thus far, the views towards developmental toxicity of BPA are controversial, numbers of studies with laboratory rodents have indicated that exposure to BPA during pregnancy and/or lactation can reduce offspring survival, birth weight, and growth in early life (NTP-CERHR, 2007). According to our previous studies, BPA could induce brain and neural tube abnormalities in rat embryos cultured in vitro (Xing et al., 2010), and inhibit the proliferation and differentiation of rat embryonic midbrain cells (Xiao et al., 2011).

Unlike BPA and other synthetic industrial products, genistein (GEN), the predominant component of phytoestrogens, is mainly derived from soybean and other legumes. It has been shown that, phytoestrogens could be of benefit in cancer prevention, osteoporosis prevention and post-menopausal syndrome attenuation (Atteritano et al., 2008; Marini et al., 2007; Warri et al., 2008). Additionally, soy formula has been serving as alternative diet for infants with milk allergy or lactose intolerance for quite a long time. Unfortunately, some studies indicated that consumption of phytoestrogens, soy-containing foods, and soy components could result in adverse effects in reproductive and developmental...
processes (Cassidy et al., 1994; McMichael-Phillips et al., 1998; NTP-CERHR, 2006; Rozman et al., 2006), though mechanism of action and effects on the developing embryo have not been fully understood.

Given that BPA and GEN are used extensively in daily life and industrial applications, it is possible that humans are exposed to them simultaneously. Although, actually according to the ECETOC (EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS, technical report No. 115, 2012), additivity is the norm and synergism the rare exception. In harness with previous studies, the potential joint toxicity of estrogenic endocrine disruptors cannot be overlooked (Kortenkamp, 2007). The use of factorial designs, in which n chemicals are studied at x dose levels (xn treatment groups), to detect interactive effects between more than two chemicals in a mixture, has been proven to be a valuable statistical approach by the US Environmental Protection Agency, for it could take full advantage of the data obtained (Groten et al., 1996).

The embryonic stem cell test (EST) was validated by the European Center for the Validation of Alternative Methods (ECVAM) to predict the embryotoxic potential of a given chemical. In the EST protocol, the inhibition of differentiation into contracting cardiomyocytes, as well as the cytotoxic effects on mouse embryonic stem cells (usually D3) and 3T3 fibroblasts cells were taken into consideration to assess embryotoxicity in vitro (Balls and Hellsten, 2002; INVITTOX Protocols No. 113). The EST method is a rapid and good approach which only involves two permanent cell lines, while other available embryotoxicity tests require a large number of pregnant animals or primary embryonic tissues and cells (Yu et al., 2008). Therefore, the embryonic stem cell test is a promising model in the detection and classification of compounds according to their teratogenic potency.

The aim of this study was to evaluate the embryotoxic potency of BPA and GEN individually in the EST model and assess the combined effects of these two compounds. A 4 x 4 full factorial design and the estimated marginal means plot were used.

2. Materials and methods

2.1. Test compounds

Bisphenol A (97% pure) and genistein (98% pure) were dissolved in DMSO (Amresco, Solon, OH ACS grade Cat. No. 67-68-5) for further evaluation. Using DMSO as the solvent control, and the final solvent concentration was 0.1%. 5-Fluorouracil (5-FU, positive control) was dissolved in PBS (Solarbio P1022500) and added to the medium. All chemicals used were of analytical grade and were obtained from Sigma (St. Louis, Mo, USA).

2.2. Cell culture

The mouse embryonic stem cell line D3 (a donation from Laboratory of Molecular and Cell Biology and the Laboratory of Stem Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.) was maintained in a pluripotent state (Fig. 1 A) in KNOCKOUT DMEM (Gibco, Cat. No. 10829018) medium supplemented with 15% ES cell-qualified fetal bovine serum (Biochrom AG Cat. No. 50615), 2 mM l-glutamine (Invitrogen, Cat. No. 25030024), penicillin/streptomycin solution (Invitrogen, Cat. No. 15140122), 0.1 mM β-mercaptoethanol (Gibco, Cat. No. 21985023), 1% (v/v) nonessential amino acid (Invitrogen, Cat. No. 11140035), and leukaemia inhibitory factor (mLIF; Millipore, Cat. No. ESG1106).

The mouse Balb/c 3T3 clone A31 fibroblast line (a donation from the Peking University Center for Human Disease Genomics, Beijing, China) was cultured in high glucose (4.5 g/l) DMEM (Gibco® Cat. No. 11965092), containing 10% heat inactivated fetal calf serum (Yuan hen 070628), 2 mM l-glutamine (Invitrogen, Cat. No. 25030024), and penicillin/streptomycin solution (Invitrogen, Cat. No. 15140122).

Before using the differentiation or cytotoxicity assay, the cells were passaged with 0.05% trypsin/EDTA solution (Gibco, Cat. No. 25300054) for a minimum of 3 times and a maximum of 20 times after thawing.

2.3. Cytotoxicity assays with mouse embryonic stem cells and 3T3 cells

Cytotoxicity assays were performed as described (INVITTOX Protocols No. 113). Briefly, on day 0, a concentration range of the test chemical covering the relevant dose–response range determined by the range-finding experiment was prepared, and 500 cells were seeded into each well of a 96-well plate in the presence of chemically modified fetal calf serum (Yuan hen 070628), 2 mM l-glutamine (Invitrogen, Cat. No. 25030024), and penicillin/streptomycin solution (Invitrogen, Cat. No. 15140122).

Before using the differentiation or cytotoxicity assay, the cells were passaged with 0.05% trypsin/EDTA solution (Gibco, Cat. No. 25300054) for a minimum of 3 times and a maximum of 20 times after thawing.

Fig. 1. Overview of embryonic stem cells (ESC) differentiation assay in vitro. (A) Day 0: the undifferentiation ESCs; (B) Day 3-Day 5: cell culture in “hanging drops” to induce the ESC aggregates; (C) Day 5-Day 10: cell culture in suspension culture system for further differentiation in “embryoid bodies” (EBs); (D) Day 10: differentiation in myocard, and the arrow indicates contracting foci of EBs. Scale bar = 500 μm.
The ES culture medium contained following concentrations of BPA: 0, 5.267, 7.901, 11.852, 17.778, 26.667, 40 and 60 μg/ml (23.1–263.2 μM); GEN: 0, 1.756, 2.634, 3.951, 5.926, 8.889, 13.333 and 20 μg/ml (6.5–49.3 μM); 5-FU as the positive one, control plates included 5-FU following concentrations of 0, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5 and 1 μg/ml (0.1–7.7 μM).

The mean OD_{490} of the blank wells was determined and subtracted from the OD_{490} values of the solvent controls and the chemically-exposed wells. The solvent controls values were set to a cell viability of 100%, and the other treated cell viability was calculated from the corresponding values. The following equation was used: percentage cell viability = (OD_{chemical-treated cells}/OD_{solvent-control cells}) × 100.

2.4. Embryonic stem cell differentiation assay

As to described previously (INVITTOX Protocols No. 113), a differentiation assay was performed to detect chemical-induced changes differentiation of pluripotent embryonic stem cells into contracting myocardial cells. In short, eight concentrations of the test chemical as well as the untreated control and the solvent control with ES cells suspension were prepared. Then, 750 cells in 20 μl of supplemented assay medium (without mLF to allow differentiation) were placed as hanging drops (Fig. 1B) on the inner side of a 100-mm Petri dish lid (50–80 drops per dish). After three days of hanging culture, the formed EBs (embryonic bodies) were transferred to the bacterial Petri dishes containing appropriate chemicals for another 2 days (Fig. 1C). On day 5 of the differentiation assay, add one EB in a small volume with 75% of the solvent control volume with cut yellow tip per well of 24 well tissue culture plates. The contracting foci were determined under light microscopy after another 5 days of culture (Fig. 1D) on the inner side of a 100-mm Petri dish lid (50–80 drops per dish). These values and related concentration were used to calculate the ID_{50} (50% inhibition of cardiomyocyte differentiation).

2.5. Statistics

Data indicates mean values ± standard deviations, and all experiments were repeated at least three times. Significance of the individual chemical measurements was determined by SPSS version 13.0 with one-way ANOVA (analysis of variance) and Dunnett’s post hoc test. The significance level was set to p < 0.05.

2.5.1. Classification of chemicals

To predict embryotoxic potential of the test chemicals, the validated prediction model (PM) was applied with three variables calculated from this in vitro test, namely, ID50 (50% inhibition of cardiac cell differentiation), IC50D3 (50% viability of ES-D3 cells), and IC503T3 (50% viability of 3T3 cells). The ID_{50}, IC_{50D3}, and IC_{50T3} were calculated based on the DoseResp function (a sigmoidal model for concentration–response curves) using Origin 8.0 software. The linear discriminant functions incorporating the three variables are presented in Table 1.

2.5.2. Prediction and assessment of combined effects

There are three types of joint effects that can be observed when two or more chemicals are applied simultaneously to a system, namely, additive effect, synergism, and antagonism (Croten, 2000). In this study, three dose levels of each compound were selected (the chosen levels were generally presumed to be below, equal to, and above the developmental toxicity threshold of the individual compound), and a 4 × 4 full factorial analysis of two-way ANOVA experimental design (Lin and Janz, 2006; Sun et al., 2009), BPA and GEN were combined at all chosen dose levels in all possible combinations (i.e., 0, 0.0625, 2.5, and 10.0 μg/ml of BPA combined with 0, 0.117, 0.469, and 1.875 μg/ml of GEN). Equivalent DMSO was added to control medium of the culture group. Then the estimated marginal means plot were used to determine the combined developmental effects of the two chemicals. An accredited approach to identify categories of combined effect is based on consideration of interaction: if the slope of dose–response curve of one chemical does not change in the presence of other chemicals, the combined effect is additive; conversely, an interaction exists, changes of the direction of the slope were used to determine the interaction is synergism or antagonism (Gennings et al., 2005).

3. Results

3.1. Embryotoxic effects of 5-Fluorouracil (Table 2)

Before the chemicals of interest were tested, the quality of the assay was checked using 5-Fluorouracil as a positive control. In terms of the cytotoxicity assay, final test concentrations of 1, 0.5, 0.25, 0.125, 0.063, 0.031 and 0.016 μg/ml (7.7–0.1 μM) of 5-FU for ES and 3T3 cells were used, while the highest concentrations of 0.08 μg/ml for the ES cell differentiation assay was tested concurrently and diluted in a 1.5-fold dilution series. The IC_{50} values were 0.056 μg/ml with 3T3 cells, and 0.098 μg/ml with ES cells. The ID_{50} calculated was 0.040 μg/ml (Fig. 2A), thus, according to the linear regression, 5-FU was classified as strongly embryotoxic (Table 5), which was consistent with previous research.
3.2. Embryotoxic effects of bisphenol A (Table 3)

To obtain the cytotoxicity data for BPA, after ten days exposed to seven concentrations of BPA covering the relevant range of “dose response” determined in the range finder experiment, the OD value of each well was measured at 490 nm for calculating the IC50D3 and IC503T3. As shown in Fig. 3B, BPA affected cell viability in a concentration dependent manner, and compound concentrations reducing cell viability to 50% (IC50) were 28.923 µg/ml for ESCs and 14.965 µg/ml for 3T3.

Concerning the differentiation assay, following the validated protocol, differentiation into contracting myocardial cells was determined by light microscopy after ten days of culture. In addition, the assay was acceptable if at least 21 out of the 24 EBs had contracting foci. BPA was tested at concentrations ranging from 0.156 to 20 µg/ml (0.06–87.7 µM) in culture medium, and 50% decrease of the number of wells containing differentiated cardiomyocytes compared to solvent controls (ID50) caused by BPA was further obtained at the concentration of 7.493 µg/ml.

According to the PM, the result of function exceeded the results of functions I and III. Therefore, BPA was classified as weakly embryotoxic (Table 5).

3.3. Embryotoxic effects of genistein (Table 4)

The cytotoxicity assays of genistein found that a concentration-dependent decrease in the cell viability of 3T3 and ESCs cells exposed to seven dilutions of compound for 10 days. Obtained from the MTS assay, and then determined by the Origin 8.0 DoseResp function, the IC50D3 and IC503T3 were 3.373 and 4.616 µg/ml respectively.

At day ten of the assay, the number of wells containing contractile EBs was recorded under the light microscope, and the cardiac differentiation potency of ES cells treated with the appropriate concentrations were shown in Fig. 2C. The ID50 of GEN calculated from the representative concentration–response curve was 1.890 µg/ml.

The three endpoints were applied as variables in the linear discriminant functions to classify GEN as a strongly embryotoxic compound, as the result of function III exceeded the results of functions II and I Table 5).

3.4. Combined embryotoxic effects of bisphenol A and genistein (Table 6)

The concentration levels were selected based on appropriate developmental toxicity of BPA and GEN treated individually (the three applied doses were presumed to be below, equal to, and above the IC50 of the individual compound), and a 4 × 4 factorial design was used to determine the combined effects of the two compounds. Results of 4 × 4 factorial ANOVA are presented in Table 7, further analysis of the related type of interaction was performed by using estimated marginal means plot.

For 3T3 cells (Fig. 3A) and D3 cells (Fig. 3B) proliferation, the overall interaction between BPA and GEN was additive as the profile plots produced approximately parallel lines.

For D3 cells differentiation (Fig. 3.C), generally, the overall interaction was significant (P < 0.001, Table 7), and the result of further interactive analysis using estimated marginal means plot was presented in Table 8. At the combination level of B0.625 group, B2.5 + G0.469, and B10 + G1.875, the differentiation of cardiac ES cells was significantly inhibited even if the concentration of BPA or GEN exposed was at NOAEL. Similar to that of individual chemical exposure, the numbers of beating EBs as well as the area of contraction were decreasing markedly with the increase of the dose.

Collectively, for ESCs proliferation and differentiation, the toxicity of BPA was enhanced by GEN, and the combined effect of developmental toxicity of BPA and GEN on differentiation of cardiac ES cells was synergistic.

4. Discussion

In this study, we used the EST model to evaluate the potential developmental toxicity of BPA, GEN and their mixtures. The embryonic stem cell test (EST) was first established as an INVITTOX protocol by ECVAM (INVITTOX Protocols No. 113) after numerous pre-validated and validated studies; it is the only method in which no pregnant animals or embryonic tissues are needed. Although one of the limitations of the EST is that the test only looks at the effect of compounds on a single endpoint, namely cardiomyocyte differentiation (Buesen et al., 2004), the EST remains the most reductionistic reflection of embryonic development of the three alternative methods provided by ECVAM.
was reported that exposure to 80

Embryotoxic classification of 5-Fluorouracil, bisphenol A and Genistein. Effect of Genistein on ES and 3T3 cell proliferation and differentiation (%,

** Indicated significant difference from DMSO control,

/C3 Indicated significant difference from DMSO control,

* Indicated significant difference from DMSO control,

Note: values are expressed as mean ± S.D.

Indicated significant difference from DMSO control,

Indicated significant difference from DMSO control,

** Indicated significant difference from DMSO control,

Note: values are expressed as mean ± S.D.

Indicated significant difference from DMSO control,

Indicated significant difference from DMSO control,

** Indicated significant difference from DMSO control,

Table 3
Effect of bisphenol A on ES and 3T3 cell proliferation and differentiation (%,

<table>
<thead>
<tr>
<th>BPA (µg/ml)</th>
<th>Proliferation</th>
<th>BPA (µg/ml)</th>
<th>Proliferation</th>
<th>BPA (µg/ml)</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3T3 cells</td>
<td>ESCs</td>
<td>ESCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.625</td>
<td>97.999 ± 1.374</td>
<td>5.267</td>
<td>104.210 ± 9.110</td>
<td>0.156</td>
<td>97.222 ± 4.811</td>
</tr>
<tr>
<td>1.25</td>
<td>96.507 ± 4.173</td>
<td>7.901</td>
<td>107.079 ± 5.225</td>
<td>0.313</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>91.897 ± 5.428</td>
<td>11.852</td>
<td>108.988 ± 6.565</td>
<td>0.625</td>
<td>97.222 ± 4.811</td>
</tr>
<tr>
<td>5</td>
<td>90.867 ± 1.448</td>
<td>17.778</td>
<td>98.988 ± 0.460</td>
<td>1.25</td>
<td>91.667 ± 8.333</td>
</tr>
<tr>
<td>10</td>
<td>83.266 ± 4.176</td>
<td>26.667</td>
<td>70.535 ± 11.945</td>
<td>2.5</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>32.770 ± 0.840</td>
<td>40</td>
<td>25.076 ± 10.662</td>
<td>5</td>
<td>91.667 ± 8.333</td>
</tr>
<tr>
<td>40</td>
<td>16.701 ± 1.193</td>
<td>60</td>
<td>16.008 ± 4.191</td>
<td>10</td>
<td>8.333 ± 8.333</td>
</tr>
</tbody>
</table>

Note: values are expressed as mean ± S.D.

Table 4
Effect of Genistein on ES and 3T3 cell proliferation and differentiation (%,

<table>
<thead>
<tr>
<th>GEN (µg/ml)</th>
<th>Proliferation</th>
<th>GEN (µg/ml)</th>
<th>Proliferation</th>
<th>GEN (µg/ml)</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3T3 cells</td>
<td>ESCs</td>
<td>ESCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.469</td>
<td>95.844 ± 11.097</td>
<td>1.756</td>
<td>101.415 ± 8.438</td>
<td>0.059</td>
<td>97.222 ± 4.811</td>
</tr>
<tr>
<td>0.938</td>
<td>96.914 ± 9.399</td>
<td>2.634</td>
<td>85.958 ± 3.917</td>
<td>0.117</td>
<td>94.444 ± 9.623</td>
</tr>
<tr>
<td>1.875</td>
<td>89.924 ± 5.534</td>
<td>3.951</td>
<td>42.637 ± 1.391</td>
<td>0.234</td>
<td>97.222 ± 4.811</td>
</tr>
<tr>
<td>3.75</td>
<td>79.549 ± 3.897</td>
<td>5.926</td>
<td>22.946 ± 3.659</td>
<td>0.469</td>
<td>94.444 ± 9.623</td>
</tr>
<tr>
<td>7.5</td>
<td>38.163 ± 3.168</td>
<td>8.889</td>
<td>13.588 ± 1.733</td>
<td>0.938</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>20.738 ± 0.541</td>
<td>13.333</td>
<td>8.908 ± 6.071</td>
<td>1.875</td>
<td>52.778 ± 12.729</td>
</tr>
<tr>
<td>30</td>
<td>18.566 ± 0.748</td>
<td>20</td>
<td>11.562 ± 2.570</td>
<td>3.75</td>
<td>0**</td>
</tr>
</tbody>
</table>

Note: values are expressed as mean ± S.D.

Table 5
Embryotoxic classification of 5-Fluorouracil, bisphenol A and Genistein.

<table>
<thead>
<tr>
<th>Chemical (µg/ml)</th>
<th>IC_{50} 3T3</th>
<th>IC_{50} D3</th>
<th>ID_{50}</th>
<th>Values of functions</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU</td>
<td>0.056 ± 0.002</td>
<td>0.098 ± 0.002</td>
<td>0.040 ± 0.003</td>
<td>−28.141 −14.409 −0.159</td>
<td>Strongly embryotoxic</td>
</tr>
<tr>
<td>BPA</td>
<td>15.545 ± 0.867</td>
<td>28.923 ± 1.825</td>
<td>7.493 ± 1.837</td>
<td>−6.280 0.707 −4.874</td>
<td>Weakly embryotoxic</td>
</tr>
<tr>
<td>GEN</td>
<td>4.616 ± 0.328</td>
<td>3.373 ± 0.175</td>
<td>1.890 ± 0.042</td>
<td>−13.056 −4.362 −2.881</td>
<td>Strongly embryotoxic</td>
</tr>
</tbody>
</table>

4.1. Developmental toxicity of bisphenol A and genistein

Currently, numerous studies provide evidence of a direct link between bisphenol A exposure during the neonatal developmental period and developmental defects (Vandenberg et al., 2007). In this study, at 5 µg/ml BPA, the differentiation of ESCs were influenced significantly, since the number of beating EBs as well as the rate and range of contraction dropped dramatically. Similarly, 0.1 µM BPA could significantly activate the mRNA expression of Pax-6, an ectodermal marker, which may interfere with the development of eyes, sensory organs and central neural and epidermal tissues derived from ectodermal tissues (Yamamoto et al., 2007). In addition, 2 µg/ml (8.8 µM) BPA could markedly reduce abalone embryo hatchability, increase developmental malformation, and suppress the metamorphosis behavior of larvae (Zhou et al., 2011). And it was reported that exposure to 80 µM BPA results in teratogenic responses of larval zebrafish, including craniofacial abnormalities and edema (Saili et al., 2013).

Combining the three end-points of the EST model, BPA was labeled as weakly embryotoxic based on the criterion mentioned above. However, according to a previous study performed by our laboratory, both WEC and micro-mass assay label BPA as non-embryotoxic. In view of deficiency of the EST model (Marx-Stoelting et al., 2009), ES cells are more sensitive to toxic agents than adult cells (Laschinski et al., 1991), besides, according to the validation study, the predictivity of EST for strongly embryotoxic chemicals was 100%, and for non-embryotoxicants and weak embryotoxicants was reported to be 72% and 70%, respectively (Wobus and Loser, 2011). Thus, the actual exposure level should be taken into consideration.

As illustrated in published reports, dietary intakes of BPA were low for all age–sex groups, with 0.17–0.33 µg/kg bw/day for infants, 0.082–0.23 µg/kg bw/day for children aged from 1 to 19 years, and 0.052–0.081 µg/kg bw/day for adults (Cao et al., 2009). And the concentration of free BPA in the blood of pregnant women could reach 22.4 ng/ml (98 nM, Padmanabhan et al., 2008). In addition, unconjugated BPA concentrations in the serum of pregnant women were 0.3–4.4 ng/ml (1.3–19.3 nM), while, in the human fetus the range was approximately 1–3 ng/ml (4.4–13.2 nM, Vandenberg et al., 2007).

In this study, the no observed adverse effect level (NOAEL) of BPA on ESCs differentiation was 5 µg/ml (21.9 µM), and concurred with our previous results, the NOAEL of BPA was 25.6 µg/ml (112.3 µM) in the WEC culture (Xing et al., 2010), and 10 µg/ml (43.9 µM) in the MM test (Xiao et al., 2011). Hence, the levels above 5 µg/ml exerting influence on embryonic stem cell cardiac
Fig. 3. Interactions between the individual chemicals illustrated by profile plots (interaction plots) with comparative marginal means of cell proliferation or differentiation. (A) proliferation of 3T3 cells, the overall interaction was additive as the profile plots produced approximately parallel lines; (B) proliferation of D3 cells, the overall interaction was additive as the profile plots produced approximately parallel lines; and (C) differentiation of D3 cells, the combined effect was synergistic at lower dosages but additive at higher concentration.
Individual and combined effect of bisphenol A and genistein on ES and 3T3 cell proliferation and differentiation (%).

<table>
<thead>
<tr>
<th>BPA + GEN (µg/ml)</th>
<th>Proliferation</th>
<th>BPA + GEN (µg/ml)</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3T3 cells</td>
<td>ESCs</td>
<td>ESCs</td>
</tr>
<tr>
<td>0 + 0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0 + 1.875</td>
<td>76.630 ± 5.042</td>
<td>95.607 ± 3.396</td>
<td>0 + 0</td>
</tr>
<tr>
<td>0 + 3.75</td>
<td>55.488 ± 6.067</td>
<td>33.028 ± 1.485</td>
<td>0 + 0.117</td>
</tr>
<tr>
<td>0 + 7.5</td>
<td>20.717 ± 3.687</td>
<td>3.456 ± 0.705</td>
<td>0 + 0.469</td>
</tr>
<tr>
<td>5 + 0</td>
<td>88.081 ± 3.615</td>
<td>85.924 ± 2.208</td>
<td>0.625 + 0</td>
</tr>
<tr>
<td>5 + 1.875</td>
<td>72.600 ± 2.531</td>
<td>59.429 ± 2.840</td>
<td>0.625 + 0.117</td>
</tr>
<tr>
<td>5 + 3.75</td>
<td>54.414 ± 5.201</td>
<td>37.079 ± 1.153</td>
<td>0.625 + 0.469</td>
</tr>
<tr>
<td>5 + 7.5</td>
<td>19.983 ± 2.804</td>
<td>6.333 ± 4.954</td>
<td>0.625 + 1.875</td>
</tr>
<tr>
<td>10 + 0</td>
<td>84.706 ± 7.446</td>
<td>74.796 ± 11.933</td>
<td>2.5 + 0</td>
</tr>
<tr>
<td>10 + 1.875</td>
<td>52.049 ± 7.212</td>
<td>50.011 ± 7.292</td>
<td>2.5 + 0.117</td>
</tr>
<tr>
<td>10 + 3.75</td>
<td>45.794 ± 5.320</td>
<td>20.467 ± 7.975</td>
<td>2.5 + 0.469</td>
</tr>
<tr>
<td>10 + 7.5</td>
<td>12.477 ± 2.329</td>
<td>2.650 ± 0.235</td>
<td>2.5 + 1.875</td>
</tr>
<tr>
<td>20 + 0</td>
<td>27.265 ± 8.959</td>
<td>8.709 ± 1.314</td>
<td>10 + 0</td>
</tr>
<tr>
<td>20 + 1.875</td>
<td>26.213 ± 8.290</td>
<td>4.114 ± 0.543</td>
<td>10 + 0.117</td>
</tr>
<tr>
<td>20 + 3.75</td>
<td>13.941 ± 3.465</td>
<td>4.423 ± 0.383</td>
<td>10 + 0.469</td>
</tr>
<tr>
<td>20 + 7.5</td>
<td>7.510 ± 2.311</td>
<td>2.726 ± 0.128</td>
<td>10 + 1.875</td>
</tr>
</tbody>
</table>

Note: Values are expressed as mean ± S.D.
* Indicated significant difference from B0 + G0 control, P < 0.05.
** Indicated P < 0.01.
† Indicated significant difference from control in corresponding group (B0 + G0 or B2.5 + G0 or B5 + G0 or B10 + G0), P < 0.05.
‡ Indicated P < 0.001.

<table>
<thead>
<tr>
<th>Table 7</th>
<th>Results of × 4 factorial analysis.</th>
</tr>
</thead>
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<tr>
<td></td>
<td>3T3 proliferation</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
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<tr>
<td>BPA</td>
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<tr>
<td>GEN</td>
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<tr>
<td>BPA + GEN</td>
<td>148.093</td>
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</table>

Table 8
Type of combined effect on ES-D3 Differentiation at Each Concentration Level.

<table>
<thead>
<tr>
<th>B0.625</th>
<th>G0.117</th>
<th>G0.469</th>
<th>G1.875</th>
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<tbody>
<tr>
<td>Synergism</td>
<td>Synergism</td>
<td>Synergism</td>
<td>Synergism</td>
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<tr>
<td>Additive</td>
<td>Synergism</td>
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<tr>
<td>Additive</td>
<td>Synergism</td>
<td>Additive</td>
<td>Synergism</td>
</tr>
<tr>
<td>B0.625, B2.5, B10 denoted the concentration levels of BPA were set at 0.625 µg/ml, 2.5 µg/ml and 10 µg/ml respectively.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G0.117, G0.469, G1.875 denoted the concentration levels of GEN were set at 0.117 µg/ml, 0.469 µg/ml and 1.875 µg/ml respectively.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

differentiation appeared to be human irrelevant because of little possibility of reaching into human embryo. In sum, BPA was not identified as a potential teratogen in our study.

At one time, the consumption of diets high in soy were recommended because they were shown to have numerous beneficial effects, including chemopreventive actions against various cancers and the alleviation of some of the adverse consequences of menopause. However, there is increasing evidence that many of these beneficial effects may not indeed be true (Adlercreutz, 2002; Messina et al., 2006; Williamson-Hughes et al., 2006; Trock et al. 2006).

The most significant is that purified GEN can produce reproductive and/or developmental toxicity in rats and mice (NTP-CERHR, 2006).

The daily isoflavone exposure level of GEN varies widely across populations and individuals (Whitten and Patisaul, 2001): estimates include an infant on soy formula (40 mg genistein/day), an average Japanese consumer (25 to 100 mg/day), a vegetarian consumer (3 mg/day), and the average British consumer (1 mg/day). Free GEN levels in the cord plasma and amniotic fluid of healthy pregnant Japanese women at delivery were shown to be 0.95 × 10⁻³–1.0 × 10⁻² µg/ml (3.51–37.3 nM) and 0.79 × 10⁻³–6.6 × 10⁻³ µg/ml (2.93–24.4 nM), respectively (Adlercreutz et al., 1999; Whitten and Patisaul, 2001).

In this study, the NOAEL of GEN was 0.938 µg/ml, which is considerably higher than the general free GEN exposure level. This finding seems to agree with the NTP’s report that GEN is unlikely to cause adverse reproductive or developmental effects in human beings.

However, for the concentrations of total GEN (free + conjugates) in humans, the serum levels of that from soy ingestion range from <1 to ~5 µM (0.27–1.35 µM) (King and Klein, 2007). Because concentrated isoflavone-containing plant extracts for use as dietary supplements as well as soy-based infant formulas have been available for decades, the concentrations of circulating isoflavones in infants consuming soy formula have been shown to have as high as 5–10 µM (1.35–2.70 µg/ml) (Setchell et al., 1997). Nevertheless, the concentrations of GEN used for studying cancer inhibition in vitro were mostly in the range of 2.7–27 µM (10–100 µM) (Sanchez et al., 2009; Ishimi et al., 1999; Suzuki et al., 2002). Thus, the maximum free GEN concentration could reach 5.4 × 10⁻² to 13.5 × 10⁻¹ µg/ml (200–500 nM) for healthy women and men who received 40–90% purified GEN (Anthony et al., 1996; Busby et al., 2002). Given that the NOAEL of GEN in this study is close to the human daily uptake levels, thus, the developmental toxicity potency of GEN cannot be neglected, and we should label GEN as a potential teratogen for regular dietary intake of GEN-containing soy product as well as the higher pharmaceutical dose for clinical purposes.

4.2. Combined effects of developmental toxicity induced by bisphenol A and genistein

With technology development, there are an increasing number of pollutants, especially synthetic chemicals that coexist in the environment, which humans are exposed simultaneously or sequentially via multiple exposure routes. Those mixtures could have a serious influence on human health that is completely different from that of their individual components. Thus, the combined action of chemicals as well as the mechanism of toxicity has been a matter of interest for more and more researchers as it could reflect the actual exposure of human daily life.

Concerning the combined effects of BPA and GEN in the present research, for ES and 3T3 cell proliferation, the predominant actions were additive (Fig. 3.A, Fig. 3.B). In the micro-mass assay (Xiao et al., 1999; Whitten and Patisaul, 2001). In this study, the NOAEL of GEN was 0.938 µg/ml, which is considerably higher than the general free GEN exposure level.
et al., 2011), the action of the combined effect on LB proliferation, LB differentiation, and MB differentiation was additive. This form of action is defined as “dose addition”, which indicates that effects of mixture may be expected or observed in response to the higher dose level while the dose for each individual component is at a level at which effects are not expected to occur (US EPA, 2000). The mechanism behind the “dose addition” may be that each of the chemicals in the mixture act in the same way, by the same mechanisms, and differs only in their potencies (Groten, 2000). In the earlier studies, MCF-7 cell culture and gene expression profile mixture of environmental estrogen suggested the combined effect of estrogenic agents is dose additive manner (Kortenkamp, 2007; Sun et al., 2009), and the results of this study are in accordance with these previous reports.

Importantly, the interaction between BPA and GEN on ES cell differentiation was synergistic at lower dosages (Fig. 4C), for instance, 0.625 µg/ml BPA and 0.117 µg/ml GEN alone had no significant effect on cardiac development, whereas the combination of 0.625 µg/ml BPA and 0.117 µg/ml GEN induced a significant reduction in the beating EBs compared to controls (Table 6). Previous studies performed by our laboratory showed in the WEC assay (Xing et al., 2010) a significant synergistic interaction between BPA and GEN for most end points (12 out of 20 tested), especially in the neural tube, vascular and olfactory systems. And studies have revealed that developmentally exposed animals were more likely to demonstrate synergistic responses to estrogenic chemicals (Steven et al., 1997; Ramamoorthy, 1997). In recent years, increasing researches have focused on “low-dose” BPA effects suggesting that BPA could induce developmental toxicity under EPA limit 50 µg/kg/day in vivo or 10–7 M in vitro (Vandenberg et al., 2007; Wetherill et al., 2007). Concerning that BPA at low exposure levels had potential toxic effects on the embryonic development, (Schwengberg et al., 2005; Xing et al., 2011), reproductive system (Cabaton et al., 2010; Holtcamp, 2011), and influence the differentiation of neural cells (Kim et al., 2009; Okada et al., 2010) through estrogen receptor-dependent or -independent pathways.

As for genistein, GEN at 10 and 50 µM protected cortical neuronal cells from oxidative stress (Sonee et al., 2004), however, some studies presented conflicting conclusions, for instance, treatment with 10 µM GEN can significantly impede cardiomyocyte differentiation of embryonic stem cells (Sato et al., 2011), and treatment of neurons with GEN at 50 or 100 µM led to cell injury (Jin et al., 2007). Collectively, although we concluded that BPA alone may not have adverse reproductive or developmental effects on human beings, given the actual daily exposure to these chemicals and that BPA and GEN do have synergetic effect at lower concentration level, these mixtures may disturb normal embryo development, which could result in birth defect and behavioral alterations later in life. Thus, toxicologists and regulators need to pay much more attention to the combined effects of endocrine-disrupting chemicals and the extrapolation to low doses in the assessment of possible health risks from exposure to chemical mixtures.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

6. Funding

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.fct.2013.08.006.

References


