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Assessment of In Vitro Assay System for Thyroid Hormone Disruptors Using Rat Pituitary GH_3 Cells

Hee Jin Kim[1], Hae Young Park[1], Jeonga Kim[1], Il Hyun Kang[2], Tae Sung Kim[2], Soon Young Han[2], Tae Seok Kang[2], Kui Lea Park[2] and Hyung Sik Kim[1]

[1]*Laboratory of Molecular Toxicology, College of Pharmacy, Pusan National University, Busan 609-735*
[2]*National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul 122-704, Korea*

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ABSTRACT. The development of *in vitro* assays has been recommended to screening and testing the potential endocrine disruptors (EDs). These assay systems focus only on identifying the estrogenic or antiestrogenic activity of EDs, whereas a few studies have been carried out to screen the thyroid hormone (TH) disruptors. The aim of this study was to evaluate a test system to detect TH disruptors using rat pituitary tumor GH_3 cells. The test system is based on the TH-dependent increase in growth rate. As expected, L-3,5,3-triiodothyronine (T_3) markedly induced a morphological change in GH_3 cells from flattened fibroblastic types to rounded or spindle-shaped types. T_3 stimulated GH_3 cell growth in a dose-dependent manner with the maximum growth-stimulating effect being observed at a concentration 1×10^{9} M. In addition, T_3 increased the release of growth hormone and prolactin into the medium of the GH_3 cells culture. Using this assay system, the TH-disrupting activities of bisphenol A (BPA) and its related compounds were examined. BPA, dimethylbisphenol A (DMBPA), and TCI-EP significantly enhanced the growth of GH_3 cells in the range of 1×10^{-5} M to 1×10^{-6} M concentrations. In conclusion, this *in vitro* assay system might be useful for identifying potential TH disruptors. However, this method will require further evaluation and standardization before it can be used as a broad-based screening tool.

Keywords: Endocrine disruptors, Pituitary GH_3 cells proliferation, Thyroid hormone, Growth hormone, Prolactin.

INTRODUCTION

Endocrine disruptors (EDs) are believed to produce not only reproductive and developmental defects but also increase the incidence of some cancers (mammary gland, testis, prostate, etc.), decrease the sperm count, and induce developmental abnormalities in wildlife and humans by disrupting the endocrine system (Colborn *et al.*, 2002; Vos *et al.*, 2000). These EDs mimic, antagonize, or enhance the biological activity of endogenous hormones (Kelce and Wilson, 1997; Gray *et al.*, 1998). Due to the increasing public concern about the problems associated with EDs, several studies have examined the possible adverse effects of EDs using both *in vivo* and *in vitro* methods (Gray and Ostby, 1998; O'Conner *et al.*, 2002). These effects involve the agonistic and antagonistic effects on the estrogen or androgen systems (Andersen et al., 2002), thyroid and adrenal disruption (Brucker-Davis, 1998), and sexual differentiation during fetal development (Bigsby *et al.*, 2003). Recently, the US Environmental Protection Agency (EPA) established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). The EDSTAC finally recommended a screening strategy for detecting endocrine-active compounds that are agonist/antagonist to the estrogen/androgen receptors, steroid biosynthesis inhibitors, or altering thyroid hormone function (O'Conner *et al.*, 2000).

Although most attention is given to the potential interactions of chemicals with the sex steroid hormone systems, it was reported that some environmental chemicals interact with the thyroid hormone (TH) system (Hohenwarter, 1994; Lans *et al.*, 1994; Miyazaki *et al.*, 2004). However, the molecular mechanisms by which environmental chemicals exert these effects are

Correspondence to: Hyung Sik Kim, Division of Molecular Toxicology, College of Pharmacy, Pusan National University, San 30, Jangjeon-dong, Geumjung-gu, Busan 609-735, Korea
E-mail: hkims@pusan.ac.kr

not fully understood. TH regulates many biological processes in the body and is essential for proper neuronal proliferation, cell migration and differentiation in a developing mammalian brain (Bernal *et al.*, 2003; Oppenheimer and Schwartz, 1997). The biological functions of TH are mainly mediated by TH receptors (TRs) located in the nucleus. These TRs belong to the nuclear hormone receptor families (Zhang and Lazar, 2000) and have ability to bind to the TH response elements (TREs) in the promoter of target genes. Currently, there are no standard methods for determining if a chemical has TH disrupting potential. As an *in vitro* model, rat pituitary tumor GH_3 cells have been used for screening TH disrupting effect (Hohenwarter *et al.*, 1996; Samuels *et al.*, 1988). Moreover, this cell line expresses intracellular TH receptors at very high levels, and they respond to the physiological concentrations of TH by proliferating.

There is a general consensus that some chemicals which interfere with TH function and homeostasis act either by inhibiting synthesis of TH, altering the serum transport proteins, or affecting the catabolism of THs (Henneman, 1986; Legrand, 1986). However, there is no data to directly support the assertion that certain environmental chemicals activate or inactivate the TH. Therefore, *in vitro* screening methods should reflect the known mechanisms of these actions. The use of GH_3 cells proliferation as a screening method might also be useful but this method requires validation and standardization before it can be used as a broad-based screening tool. Different classes of environmental chemicals including bisphenol A (BPA) and brominated flame-retardants were also examined. BPA, which is an industrial substance for polycarbonate and epoxy resins, is well known to have TH-like activity (Kitamura *et al.*, 2005). However, the thyroid hormonal activity of halogenated or methylated derivatives of BPA has not been fully examined. The aim of this study was to evaluate a test system to detect TH distuptors using rat pituitary GH_3 cells.

MATERIALS AND METHODS

Chemicals

The L-3,5,3-Triiodothyronine (T_3), tyroxine (T_4), bisphenol-A (BPA), tetrabromobisphenol A (TBBPA), dimethylbisphenol A (DMBPA), tetramethylbisphenol A (TMBPA), TCI-GR, TCI-EP and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The Dulbecco's Modified Eagle's Medium (DMEM)/F12, fetal calf serum (FCS), penicillin-streptomycin and 0.25% trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, U.S.A.). All the stock solutions were stored at 4°C and diluted to the desired concentrations in phenol red-free medium for testing. The final DMSO concentration in the medium did not exceed 0.2%.

Cell culture

The rat pituitary cell line GH_3, which was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) was maintained in phenol-red free DMEM/F12 supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. For the thyroid hormone-responsive GH_3 cell proliferation assay, the cells were grown in the test medium containing 10% T_3-depleted charcoal-dextran treated FCS (CD-FCS). Finally, the supernatant was cleared and sterilized by filtration.

Cell proliferation assay

The GH_3 cells were cultured in a humid atmosphere at 37°C and 95% air/5% CO_2 in DMEM/F12 supplemented with 10% FCS and 15 mM HEPES. The cells were sub-cultured once a week in new 25 cm^2 culture flasks. Prior to the assay, the cells were incubated for 48 h in serum free DMEM/F12 medium in order to deplete the cells of THs. Briefly, the GH_3 cells were seeded in 96-well plates containing 10% CD-FCS at an initial concentration of 2,500 cells/well. The following day the test chemicals were diluted to the required concentrations in the test medium and added to the cells. The final solvent concentration did not exceed 0.1%. The cells were incubated for a further 5 days, and cell growth was then measured by staining the fixed cells by sulforhodamine-B (SRB) dye. Briefly, the medium in each well was discarded (or used for cytotoxicity test) and the cells were fixed with cold 10% trichloroacetic acid at 4°C for 30 min. After further washes with PBS and air drying, the cells were stained with 0.4% (w/v) SRB in 1% acetic acid for 10 min. After another four washes with acetic acid, the cell-bound dye was dissolved by adding of 200 μl Tris base (10 mM, pH 10.5) and with constant shaking for 20 min. Aliquots (150 μl) from each well were then transferred to another microtiter plate to measure the absorbance at 490 nm with a reference at 630 nm using a microplate reader.

Cytotoxicity test

The cytotoxicity assay was performed within the proliferation assay. After incubating the cells with the test compounds in the proliferation assay, the culture was continued for 5 days, and the MTT assay was then car-

ried out to measure the cytotoxicity. The cells were treated with the MTT solution (5 mg/ml) and further incubated for 4 hr. One hundred ml of supernatant was replaced with the same volume of DMSO, and absorbance was read at 570 nm using a microplate reader.

Growth hormone and prolactin levels

The cells were seeded in 24-well plates at 1×10^4 cells/well and the test compounds were added on the next day. Two days later, the growth hormone levels in the culture medium were measured using a radioimmunoassay kit (RGH-45K, Linco), and the prolactin level was measured using a rat prolactin radioimmunoassay kit (Amersham, Little Chalfont, UK) according to the manufacture's protocols.

Statistical analysis

The data for each assay are relative to the solvent control, which was set to 100%, is presented as the means ± standard deviation (S.D.) of at least three independent assays. The statistical significance was evaluated by a Student's *t*-test. A $p < 0.05$ was considered significant.

RESULTS

The effects of thyroid hormones on the morphology and growth of rat pituitary tumor GH_3 cells were examined using medium containing serum depleted of thyroid hormones. As shown in Fig. 1, the GH_3 cells were polygonal in shape and were flattened in the medium containing CD-FCS, as previously reported by Hohenwarter *et al.* (1996). However, the cells treated with T_3 changed to a more rounded morphology. Therefore, T_3 induced a morphological change in the GH_3 cells from a flattened fibroblastic type to rounded type morphology. The morphological changes might be useful for evaluating the biological effects of T_3 and its analogs as well as in examining the mechanism of the thyroid hormone activity. With the morphological changes, the T_3-induced cell growth rate increased approximately 2-fold compared with the control. The growth-stimulating effect showed the same analog specificity and dose dependency as the morphological changes.

T_3-induced GH_3 cells proliferation is shown in Fig. 2. The proliferation of GH_3 cells was estimated after a 5-day treatment with T_3 (10^{-12} to 10^{-7} M). T_3 dose-dependently stimulated GH_3 cell proliferation with the maximum effect being observed at 1×10^{-9} M concentration, which is approximately 4.8 times that of the control. Concentrations above 1×10^{-8} M caused a decrease in cell growth, and the half maximum response (ED_{50}) of

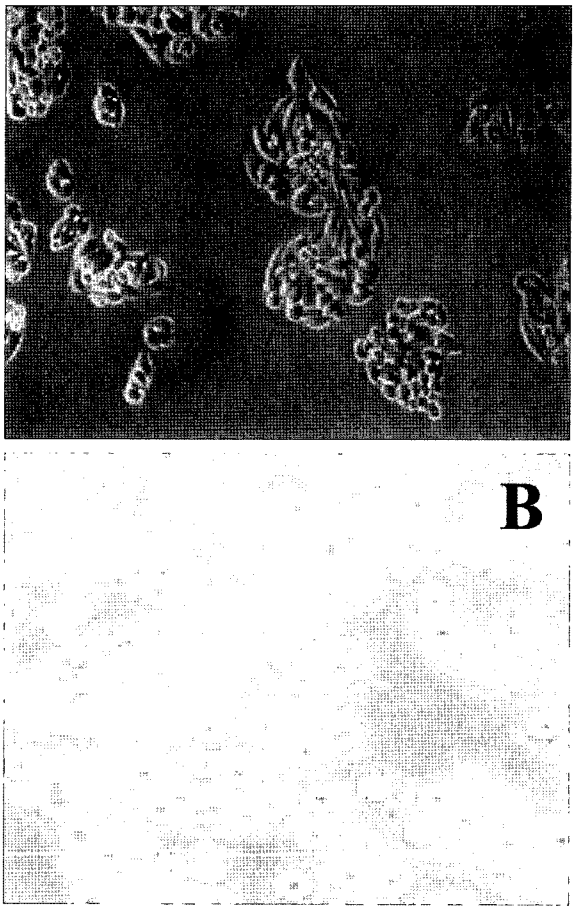

Fig. 1. Effect of the different culture media on the GH_3 cell morphology. A) DMEM supplemented with 5% CD-FCS (stripped serum) and B) serum-free DMEM medium supplemented with 0.1 nM T_3.

T_3 was determined to be 5.6×10^{-10} M. Therefore, the optimal working range of T_3 for this assay system ranged from 0.1 to 1.0 nM. The time-dependent effect of GH_3 cell proliferation was determined at 1.0 nM T_3 for 5 days. There was no increase in GH_3 cells growth after 2 days incubation but GH_3 cells growth was increased significantly after 3 days. However, the presence of CD-FCS in the culture media did not further increase the levels of GH_3 cell growth after 5 days (Fig. 3).

The effect of the TH on the release of growth hormone and prolactin was measured in a GH_3 cell culturing media. The serum growth hormone and prolactin levels were calculated using radioimmunoassay. In the control condition, the growth hormone level was approximately 5.5~5.9 nmol/ml media, and T_3 significantly increased the growth hormone levels to approximately 8.2~9.1 nmol/ml media at 1×10^{-9} M. However, T_4 did not show any significant effect (Fig. 4). Similar to growth hormone, T_3, at 1×10^{-9} M, significantly increased the

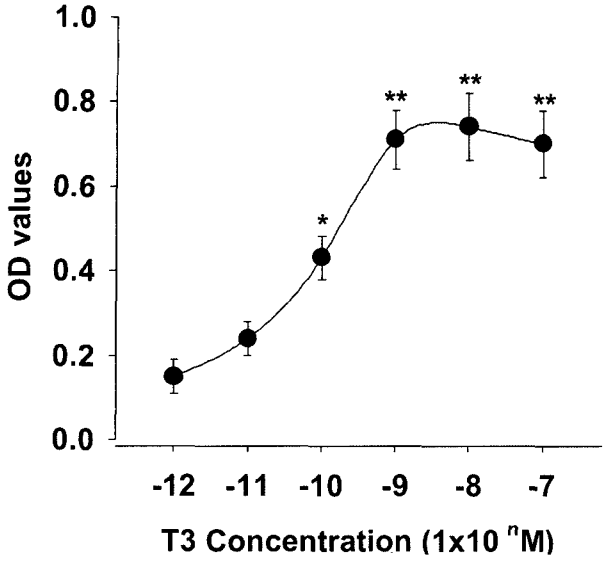

Fig. 2. Dose-response relationship of T_3 on rat GH_3 cell proliferation in media supplemented with 5% CD-FCS. The cells (2,500 cells/well) were plated in presence of T_3 and were maintained in CD-FCS medium for 5 days. The error bars were calculated as the SD, and the data is presented as a mean from triplicate experiments. $^*P < 0.05$ was considered significant. $^{**}P < 0.01$ was considered significant.

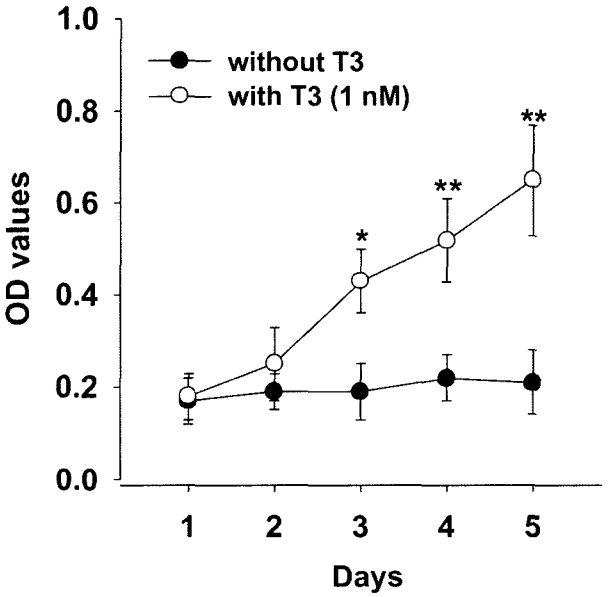

Fig. 3. Time-course relationship of T_3 on rat GH_3 cell proliferation. The cells were plated in presence of T_3 (2,500 cells/well) and maintained in CD-FCS medium for 5 days. The error bars were calculated as the SD, and the data is presented as a mean from triplicate experiments. $^*P < 0.05$ was considered significant. $^{**}P < 0.01$ was considered significant.

prolactin levels to about 2-fold compared with control, whereas the T_4 treatment maintained the release of prolactin in the medium at levels similar to those of the controls (Fig. 5).

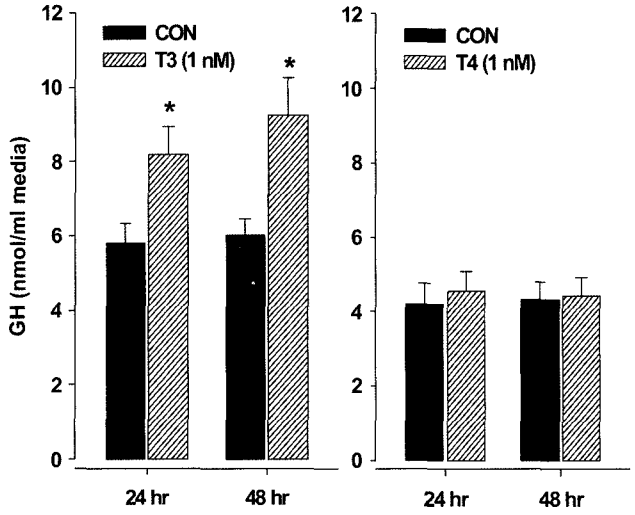

Fig. 4. Effects of T_3 and T_4 on growth hormone secretion. The cells (1×10^4 cells/well) were plated in phenol red free media supplemented with CD-FCS containing T_3 (1×10^{-9} M) and T_4 (1×10^{-9} M) for 2 days. The error bars were calculated as the SD, and the data is presented as a mean from triplicate experiments. $^*P < 0.05$ was considered significant.

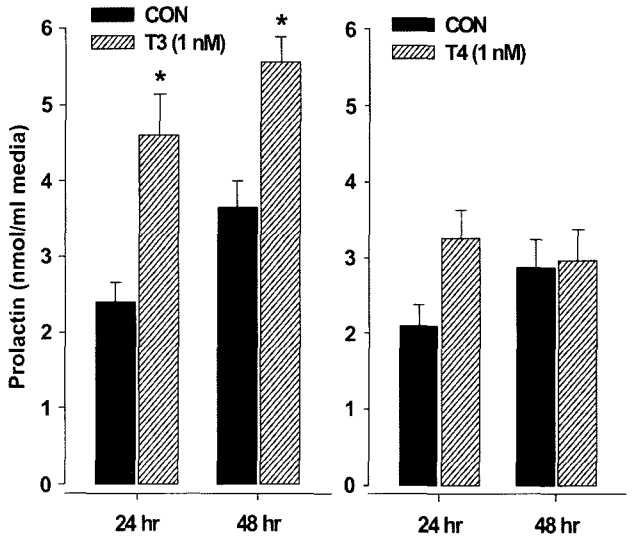

Fig. 5. Effects of T_3 and T_4 on prolactin secretion. The cells (1×10^4 cells/well) were plated in phenol red free media supplemented with CD-FCS containing T_3 (1×10^{-9} M) and T_4 (1×10^{-9} M) for 2 days. The error bars were calculated as the SD, and the data is presented as a mean from triplicate experiments. $^*P < 0.05$ was considered significant.

The thyroid hormonal activity of BPA and its related compounds was examined using GH_3 cell proliferation assay. When BPA, DMBPA, and TCI-EP were added to the culture media, growth of GH_3 cells was stimulated

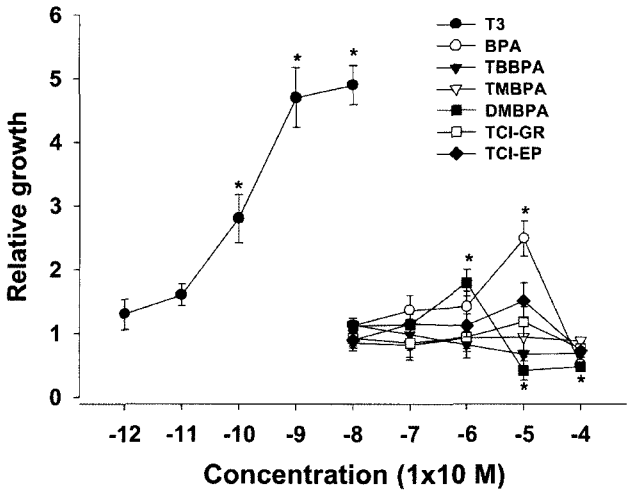

Fig. 6. Effects of bisphenol A and related compounds on GH_3 cell proliferation. The cells (2,500 cells/well) were plated in CD-FCS medium supplemented with various concentrations of chemicals for 5 days. Each bar represents the mean ± SD of triplicate experiments. Activity was expressed relative growth to the control untreated GH3 cells. $^*P < 0.05$ was considered significant.

at 1×10^{-5} M in the case of BPA and TCI-EP, and at 1×10^{-6} M in the case of DMBPA (Fig. 6). These results indicate that BPA, DMBPA, and TCI-EP show TH-like activity. However, TBBPA, TMBPA, and TCI-GR did not show agonist action on GH_3 cell growth proliferation at any concentration (Fig. 6). The MTT test was used to assess the cytotoxic response of the GH_3 cells to BPA and its related compounds. No cytotoxicity was detected at 1×10^{-5} M concentration of the test substances (Data not shown).

DISCUSSION

There is increasing evidence suggesting that a large number of EDs have the potential ability to disrupt the TH function (Brueker-Davis, 1998; Porterfield, 2000). GH_3 cells are dependent on the thyroid hormone for their proliferation in a monolayer culture (Gutleb *et al.*, 2005; Hohenwarter *et al.*, 1996; Samuels *et al.*, 1988) and possess all the necessary factors needed to measure the biological endpoint of growth. Therefore, the effect of chemicals on GH_3 proliferation should also include interactions and cross-talk between the TH actions and other cellular pathways. It was reported that THs play an important roles in the development and energy metabolism, and affect several levels of the homeostatic control (Legrand, 1986). Disturbances in the function and metabolism of TH may lead to abnormal development, altered growth patterns, and a variety of physiological perturbations in mammals (Leung *et al.*, 1990; Zoeller *et al.*, 2002). As a result, there is an increasing need for test systems to detect and quantify the potential TH disrupting effects of environmental chemicals as well as newly developed chemicals.

This study evaluated a new test method for detecting potential TH disrupting compounds. The assay used a rat pituitary tumor GH_3 cell line, the growth of which is dependent on the T_3. Generally, the growth stimulatory effect of T_3 on GH_3 cell was mediated by TRs that bind the THs to thyroid hormone-responsive elements (TREs) in the cell nucleus, ultimately leading to gene expression (Hinkle and Kinsella, 1986). Previous reports have indicated that the thyroid TH disrupting activities are based on the capacity of xenobiotics to interfere with the binding of TR. Several poly chlorinated biphenyls (PCBs) and poly brominated diphenyl ethers (PBDEs) as well as BPA and some of its derivatives were tested and shown to be active in this TR-binding assay (Brouwer *et al.*, 1996; Gauger *et al.*, 2004; Iwasaki *et al.*, 2002; Kitamura *et al.*, 2002). Based on these results, the interaction between xenobiotics and the TRs may have agonistic effects on cell growth, whereas binding of antagonists to the TRs may have inhibitory effects on T_3 mediated cell growth. Therefore, the GH_3 cells proliferation assay provides the possibility of detecting both agonists as well as antagonists at the level of TR-functioning. In this study, the proliferation of GH_3 cells was examined after 5 days of treatment with T_3. T_3 dose-dependently stimulated cell proliferation with an estimated half maximum response of 5.6×10^{-10} M. In the time-dependent effect of GH_3 cells proliferation, there was no increase in GH_3 cells growth after 2 days incubation but GH_3 cells growth had increased significantly after 3 days. Therefore, the GH_3 cells proliferation assay can be used as a reliable method for screening the T_3-like activity.

The effects of BPA and its derivatives on the estrogenic action have been well elucidated, whereas only a few *in vitro* studies have been reported the TH disrupting activity (Kitamura *et al.*, 2005; Darnerud, 2003). Recently, an *in vivo* study showed that maternal exposure to BPA in rats could elevate the serum T_3 level in nursing pups and up-regulate the expression of the TH-responsive gene, RC3/neutogranin, in the brain (Zoeller *et al.*, 2004). Moriyama *et al.* (2002) reported that BPA binds to TR and suppresses the transcriptional activity by recruiting nuclear receptor corepressors (N-CoRs) to the promoter. In this study, BPA induced significantly GH_3 cells proliferation at 1×10^{-5} M but significantly reduced at high concentration (1×10^{-4} M). This result suggests that BPA exhibits EDs effect via TH-like activ-

ity, whereas high dose of BPA significantly inhibited the GH3 cells proliferation by cytotoxicity rather than act as a TH-antagonist.

The brominated derivatives of BPA, TBBPA, TMBPA, and DMBPA, which are widely used as a flame-retardant, have been detected in wildlife and humans (Darnerud, 2003). These brominated derivatives of BPA have some structural resemblance to T_4, and it has been shown to be an effective binder to the human TTR a TH-binding protein *in vitro*, with almost 10 times higher potency than T_4 (Meerts *et al.*, 2000). A recent study reported that TBBPA and DMBPA inhibited the binding of T_3 to TR and induced the expression of rat growth hormone gene and GH_3 cell proliferation (Kitamura *et al.*, 2002). In the present study, we found that DMBPA and TCI-EP show TH-like activity. DMBPA (1×10^{-6} M) and TCI-EP (1×10^{-5} M) stimulated GH_3 cell growth but this proliferation rate was significantly inhibited at high concentrations (1×10^{-5} M or 1×10^{-4} M). There was no significant effect on GH3 cells proliferation observed by TBBPA, TMBPA, and TCI-GR treatment. It is interesting that when BPA have tetrachloro or tetrabromo substitution, their TH-like activity is markedly decreased in this assay system. These results suggest that the BPA and its halogenated compounds had low potency compared with T_3 and potential disrupting activity on the steroid hormone function including TH-dependent growth of GH_3 cells.

In conclusion, this study evaluated a new screening method for detecting TH disrupting chemicals, which might useful for detecting the EDs. However, further research will be needed to standardize or validate these results through inter-laboratory comparisons.

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