Mutual synergistic toxicity between environmental toxicants: A study of mercury chloride and 4-nonylphenol

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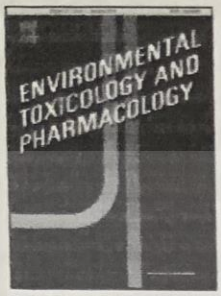

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Mutual synergistic toxicity between environmental toxicants: A study of mercury chloride and 4-nonylphenol

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ABSTRACT

Mercury chloride ($HgCl_2$) and 4-nonylphenol (NP) are widespread environmental and industrial pollutants that are known to have toxic effects as well as endocrine disrupting activities. Although the individual effects of $HgCl_2$ and NP in liver have been relatively well recognized, little is known about the interaction of NP and $HgCl_2$ during the induction of their toxicity. In the current study, we investigated the synergism between $HgCl_2$ and NP using HepG2 cells. Surprisingly, the concurrent treatment of HepG2 with $HgCl_2$ and NP induced a significant cytotoxicity at concentrations where neither of them have any cytotoxic effect when treated alone. The cytotoxicity of NP is enhanced in the presence of $HgCl_2$ (a shift from 74.9 to 47.4 μM in LC_{50}) and vice versa (a shift from 94.9 to 66.3 μM in LC_{50}). Estrogen receptor antagonists such as ICI 182,780 did not protect HepG2 cells from these cytotoxic insults. Whereas the intracellular level of reduced form glutathione (GSH) was considerably decreased upon the co-treatment with NP and $HgCl_2$. Furthermore, the synergistic cytotoxicity was significantly inhibited by 20-mM *N*-acetylcysteine (NAC). These results indicate that the mutual synergistic cytotoxicity of $HgCl_2$ and NP on HepG2 cell is not associated with estrogen receptor signaling but mediated by reactive oxygen species (ROS) generation. In our real life, we are continuously and often simultaneously exposed to many different kinds of environmental pollutants. The present study suggests a mechanism of potential synergistic adverse effects of these toxic pollutants.

1. Introduction

4-Nonylphenol (NP) is an organic compound of the wide families of alkylphenols. It is a product of industrial synthesis formed during the alkylation process of phenols, particularly in the synthesis of polyethoxylate detergents. Alkylphenol polyethoxylates (APEs) are widely used as components of detergents, paints, herbicides, insecticides, and many other synthetic products (Platt, 1978; Junk et al., 1974). Approximately 80% of APEs are NP polyethoxylates (Verslycke et al., 2005). Because of their man-made origins, NPs are classified as xenobiotics and suspected to be an endocrine disruptor capable of interfering with hormonal system (Soares et al., 2008). The estrogenic effect of NP relative to 17β-estradiol (E2) was shown to be 0.000009 in an in vitro assay using rainbow trout hepatocytes (Jobling and Sumpter, 1993). NP induces both cell proliferation and progesterone receptor expression in human estrogen-sensitive MCF-7 breast tumor cells. NP also triggers mitotic activity in luminal endometrial epithelium of ovariectomized rats, confirming the reliability of the MCF-7 cell proliferation bioassay (Soto et al., 1991).

A number of toxic heavy metals, such as mercury (Hg), are widely used in industry and are present at high levels in the environment. Although exposure to these metals is associated with several adverse health effects in humans, they are commercially important in many industries, and their occupational and environmental exposures continue to occur (Jarup, 2003). Numerous studies on mercury toxicities have revealed that it generates oxygen radicals and has a great affinity for SH groups of biomolecules, thus depleting intracellular thiols including reduced glutathione (GSH) (Hansen et al., 2006). Although the exact mechanisms of free-radical generation is not yet completely understood, it is postulated that the antioxidant GSH depletion by mercury may be a trigger for the production of reactive oxygen species (ROS) that induce lipid, protein, and DNA oxidation. Generation of ROS in the cytoplasm of cells may increase the mitochondrial hydrogen peroxide production and lipid peroxidation of mitochondrial membrane, resulting in loss of membrane integrity and finally cell necrosis or apoptosis

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(Kaur et al., 2006; Jezek and Hlavata, 2005; Oh et al., 1997; Valko et al., 2006).

4-Nonylphenol was metabolized in the liver, with the majority of the metabolites excreted in bile, mainly as glucuronide conjugates and hepatic cytochrome P450 metabolites as well (Lee et al., 1998). The uptake of mercury shows that kidney and liver accumulate the highest level of mercury compared to other organs (Hussain et al., 1999). Many of these reports indicate that both NP and $HgCl_2$ are metabolized and accumulated in liver.

However, little is recognized about the interaction of NP and $HgCl_2$ during the induction of their toxicity in liver. We were interested in any potential synergistic adverse effect of NP and $HgCl_2$ and investigated the interaction of NP and $HgCl_2$ using the human liver origin cell line HepG2 cells for the present study. To elucidate the mechanism of their action, we tested ER antagonist (ICI 182,780) whether the damage to cells induced by the synergy was mediated by estrogen receptor (ER). Additionally, we also examined if ROS play a role in the synergistic cytotoxicity by measuring intracellular GSH levels and observed protection by *N*-acetylcysteine (NAC), a cell permeable ROS scavenger.

2. Materials and methods

2.1. Chemicals and reagents

4-Nonylphenol with 98% analytical standard was from Supelco Inc. (Bellefonte, PA, USA). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin sulfate, trypsin, dimethyl sulfoxide (DMSO), propidium iodide (PI), mercury chloride, *o*-phthalaldehyde, and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). ICI 182,780 was purchased from Tocris Cookson Ltd. (Avonmouth, UK).

2.2. Cells and culture conditions

Human hepatocellular carcinoma cell line HepG2 was purchased from Korean Cell line bank. HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Treatment and cell viability assay

HepG2 cells were seeded in 12-well plates at a density of 4 × 10(4) cells/well in 1 ml DMEM containing 10% FBS overnight. Non-adherent cells were removed by gentle washing. Then cells were treated for 24 h with various concentrations of $HgCl_2$ and NP in the presence or absence of 20 mM of NAC or 1 μM of ICI 182,780 which were added 1 h before. To assess cell viability, 200 μl of MTT solution (5 mg/ml) was added to each well and incubated for another 4 h at 37 °C. After the removal of supernatant, the generated formazan crystal was dissolved by adding 200 μl/well of DMSO and the absorbance was detected at 540 nm using ELISA Reader (Bio-Rad, USA). The percentage of cell viability was calculated as follows: cell viability (%) = $absorbance_{540}$ of experimental well/$absorbance_{540}$ of control well × 100.

2.4. Determination of cellular glutathione

The level of cellular glutathione was determined by the method of Hissin and Hilf (1976), with a modification made to the assay for its use in cell culture studies. Briefly, HepG2 cells in P60 dishes (about 50–70% confluence) were incubated for 24 h with either vehicle alone or testing compounds as indicated in the figures. The treated cells were harvested by trypsinization and centrifugation followed by washing with PBS. The cells were resuspended in 375 μl of ice-cold phosphate–EDTA buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8.0, prepared daily) and sonicated (30 s) on ice for three times in 1 min intervals. The protein in the cell extract was precipitated by the addition of 100 μl of 25% HPO_3. The total homogenate was centrifuged at 100,000 × *g* at 4 °C for 30 min and the resulting supernatant was used for the determination of cellular glutathione content. For the glutathione assay, 100 μl of the 100,000 × *g* supernatant was added into 0.9 ml of the phosphate–EDTA buffer for dilution. The final assay mixture (2.0 ml) contained 100 μl of the above-diluted supernatant, 1.8 ml of phosphate–EDTA buffer, and 100 μl of the *o*-phthaldialdehyde (OPT) solution (containing 100 μg of OPT in reagent-grade absolute methanol). After thorough mixing and incubation at room temperature for 15 min, the fluorescence was determined (excitation of 350 nm, emission at 420 nm) using a fluorometer.

2.5. Statistics

The results are expressed as mean standard deviation (S.D.). A paired Student's *t*-test was used to assess the significance of differences between two mean values. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Cytotoxic effects of $HgCl_2$ or 4-NP

To examine the toxic effects on hepatic tissue of $HgCl_2$ and NP, we treated the human liver origin cell line HepG2 with $HgCl_2$ or NP as described in Section 2. Briefly, HepG2 liver cells were incubated in the absence or the presence of either $HgCl_2$ or NP for 24 h (Fig. 1). As a result, NP significantly reduced cell viability at concentrations of 60 μM and higher. $HgCl_2$ meaningfully decreased cell viability at concentrations of 90 μM and higher. No significant difference was observed at low concentrations of NP (1–50 μM) and $HgCl_2$ (1–80 μM) relative to the control group.

3.2. Synergistic effect of $HgCl_2$ and NP

Although the effects of $HgCl_2$ or NP in liver have been recognized well, little is known about the interaction of NP and $HgCl_2$ on the induction of toxicity. For evaluating any possible synergism between the two compounds, we chose no effect concentrations of NP (50 μM) and $HgCl_2$ (80 μM). It was a quite surprising that the results showed a dramatic increase of cell death in simultaneous treatment of HepG2 with $HgCl_2$ and NP, comparing with neither of them has any cytotoxic effect when treated alone (Fig. 2A). When

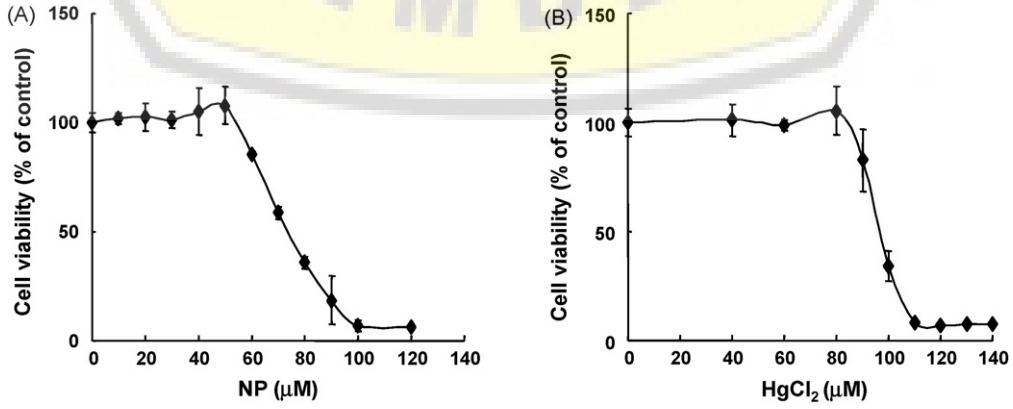

Fig. 1. Effects of NP or $HgCl_2$ on the viability of HepG2 cells. Cells were treated for 24 h with the indicated concentrations of (A) 4-nonylphenol or (B) mercury chloride. Cell viability was assessed by MTT assays. Data are presented as the percentage of respective controls. Cell viability was reduced in a concentration dependent manner by mercury chloride or 4-nonylphenol exposure. Results are expressed as mean ± S.D. for 3 independent experiments which were separately performed in triplicate.

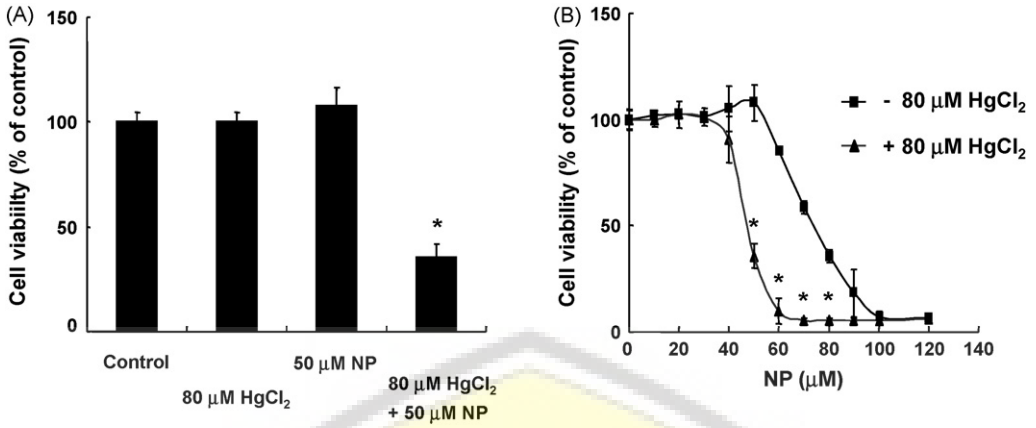

Fig. 2. Mutual synergistic cytotoxicity of $HgCl_2$ and NP in simultaneous treatment. (A) Cells were incubated with either 80 μM of mercury chloride or 50 μM of 4-nonylphenol individually or co-incubated with the both simultaneously. Co-treatment of HepG2 with $HgCl_2$ and NP induced a significant cytotoxicity at concentrations where neither of them have any cytotoxic effect when treated alone. *$P < 0.01$ vs. control. (B) Cells were treated with indicated concentrations of NP in the presence or absence of 80 μM mercury chloride. Cell viability was assessed by MTT assays. Results are presented as mean ± S.D. for 3 independent experiments performed separately in triplicate. *$P < 0.01$ vs. respective controls (−$HgCl_2$).

HepG2 cells were incubated with the indicated concentrations of NP, the cytotoxicity of NP was greatly enhanced in the presence of $HgCl_2$ comparing with that in the absence $HgCl_2$ (a shift from 74.9 to 47.4 μM in LC_{50}) (Fig. 2B). The reverse was also true (data not shown). Furthermore, we could observe morphological changes of HepG2 cells only when they were simultaneously exposed to $HgCl_2$ and NP but not in the individual treatment (Fig. 3). The co-treated HepG2 cells showed a more rounded types of morphology comparing with others.

3.3. *Effects of estrogen receptor inhibitors against $HgCl_2$ and NP-induced cell death*

HepG2 cells are generally known to be estrogen responsive although the ERα level is low (Marino et al., 2001). In addition, both $HgCl_2$ and NP are suspected to have estrogenic activity as endocrine disruptors though their activities are relatively weaker than endogenous estradiol. Therefore, we could not exclude the possibility of involvement of estrogenic signal by the agents in the synergistic adverse effect of our present results. To investigate whether damage to cells induced by synergistic effect was mediated by ER, we pretreated cells with ER antagonist (ICI 182,780) 1 h prior to treatment with $HgCl_2$ and/or NP. From the results, ER antagonist did not abolish mutual synergistic toxicity in HepG2 cells (Fig. 4). This result suggests that not ER signaling but another molecular mechanism may be implicated with hepatocyte damage synergistically induced by $HgCl_2$ and NP simultaneous treatment.

3.4. *Synergistic mechanism of $HgCl_2$ and NP-induced cytotoxicity by generating reactive oxygen species*

It has been previously proposed that $HgCl_2$ or NP may deplete the body's content of reduced GSH and increase free-radical load. Therefore, we have tested whether ROS play a role in co-effect of $HgCl_2$ and NP-induced cell death of the present study. There was a mild decrease of GSH levels in cells exposed to NP (50 μM) or $HgCl_2$

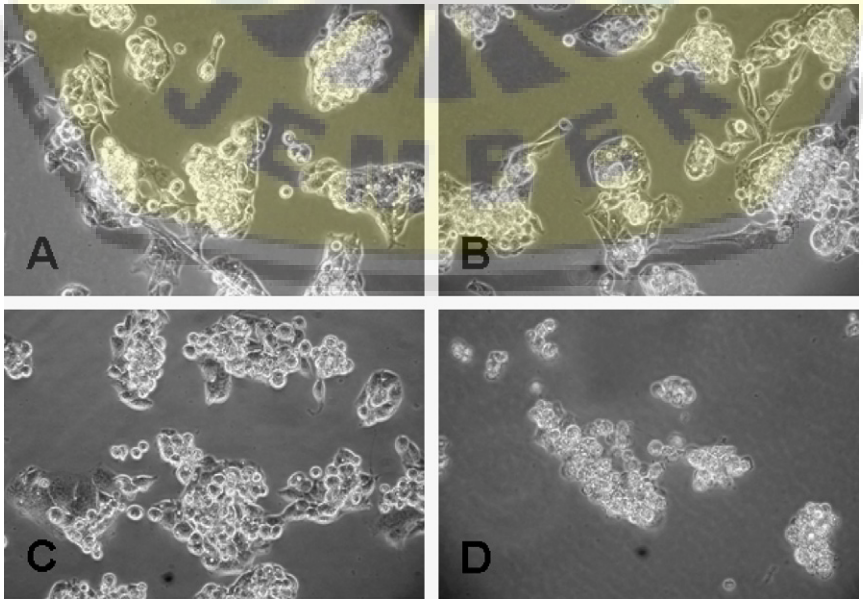

Fig. 3. Morphological changes of HepG2 cells observed upon the treatment with $HgCl_2$ and/or NP. HepG2 cells were incubated for 24 h in (A) the absence or the presence of either (B) 80 μM of $HgCl_2$ or (C) 50 μM of NP, respectively or (D) the both simultaneously. The treated cells were examined and photographed using light microscopy.

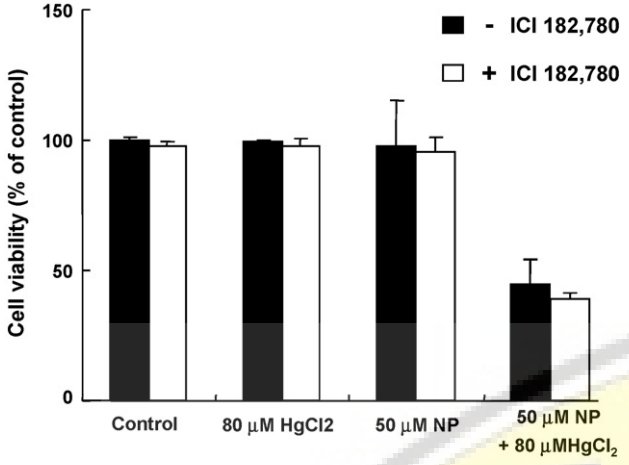

Fig. 4. Estrogen receptor antagonist ICI 182,780 does not protect HepG2 cells from the mutual synergistic cytotoxicity of $HgCl_2$ and NP. HepG2 cells were pretreated with ICI 182,780 for 1 h, then challenged to $HgCl_2$ and/or NP for additional 24 h. Cell viability of each treatment was assessed using MTT assay. Results are expressed as mean ± S.D. for 3 independent experiments.

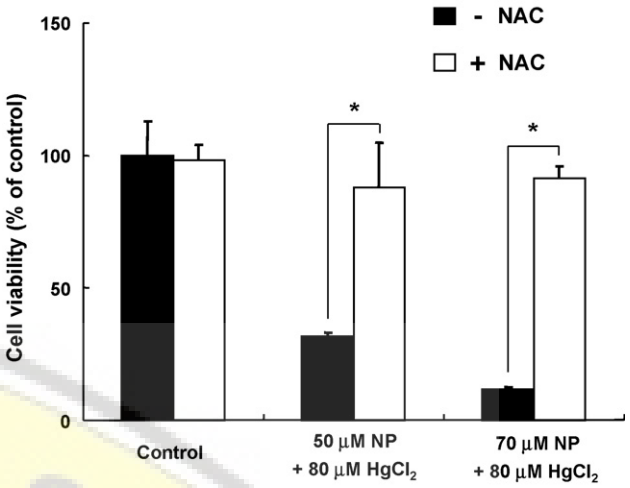

Fig. 6. Pretreatment of NAC protects HepG2 cells from $HgCl_2$ and NP-induced cytotoxicity. HepG2 cells were incubated in the presence or absence of 20 mM NAC for 1 h, then challenged to $HgCl_2$ and 4-nonylphenol simultaneously for additional 24 h. Cell viability of each treatment was assessed using MTT assay. Results are expressed as mean ± S.D. for 3 independent experiments. *$P < 0.01$ vs. respective controls (−NAC).

(80 μM), however, the co-treatment of HepG2 cells with $HgCl_2$ and NP resulted in a marked decrease in the intracellular level of GSH (Fig. 5). This result indicates that ROS may have a pivotal role in $HgCl_2$ and NP-induced toxicity of HepG2 cells. To confirm this, we tested NAC, a cell permeable ROS scavenger, for removing the oxidative stress imposed to HepG2 cells upon the co-treatment. Pretreatment of cells with NAC resulted in a significant inhibition of the cytotoxicity comparing with their respective controls (−NAC) (Fig. 6). These results suggest that the mutual synergistic cytotoxicity of $HgCl_2$ and NP are induced by the mechanism of generation of reactive oxygen species, which can be excessively escalated in a situation when a group of toxic substances are present together simultaneously.

4. Discussion

Humans are being continuously and often simultaneously exposed to various toxic substances, including mercury and NP, by means of either their occupational or environmental exposures.

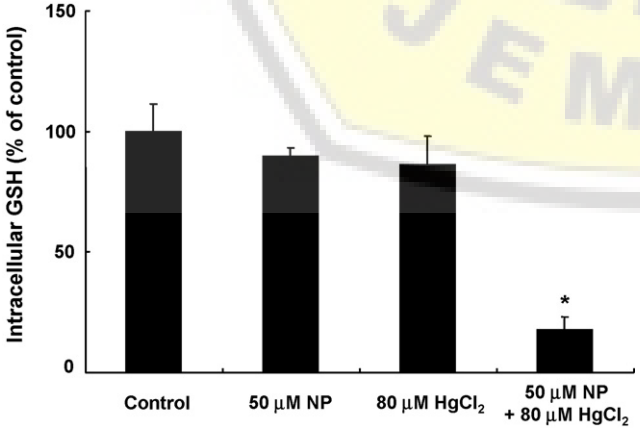

Fig. 5. Effect of $HgCl_2$ and/or NP on the level of intracellular reduced GSH. Fluorometric method using OPT solution was used to measure GSH as described in Section 2. Results represent the average of three different experiments. GSH was calculated as nmole of GSH per mg of protein and then was presented as the percentage of control group. Results are expressed as mean ± S.D. for 3 independent experiments. *$P < 0.01$ vs. control.

Inorganic form of mercury, such as elemental mercury is generally poorly absorbed from the gastrointestinal tract; however mercuric salts can be absorbed from GI and distributed predominantly to kidneys, and also other vital organs, including liver, spleen and central nervous system. The major target organs of inorganic mercury are kidney and liver (Holmes et al., 1992; Zalups, 2000; Sanchez et al., 2001). NP, an endocrine disrupter, tends to persist in liver tissue, where it is metabolized mainly as glucuronide conjugates and hepatic cytochrome P450 metabolites. NP has been reported to induce centrilobular liver cell hypertrophy (Woo et al., 2007). In our study, cytotoxicities have been consistently observed at threshold concentrations of 50 μM of NP and 80 μM of $HgCl_2$ in HepG2, respectively. However, no cell death has been detected in HepG2 at the lower concentrations. To examine any possible synergistic interaction between NP and $HgCl_2$, we selected no cytotoxic concentrations of NP (50 μM) and $HgCl_2$ (80 μM) for the present work. Consequently, we could observe a dramatic increase of cell death in simultaneous treatment of HepG2 with $HgCl_2$ and NP, comparing with neither of them has any cytotoxic effect when treated alone (Fig. 2). Upon the simultaneous exposure to $HgCl_2$ and NP, we could observe morphological changes of HepG2 hepatic cells (Fig. 3). Initially, the cells showed decrease of cell size, followed by cell rounding up. Apparently, it appears that many of these cells eventually go to cell round up, which is presumably a part of process to apoptotic cell death. Recently, the risk has been suggested that each chemical alone does not show effects from a small dose administration, but appears efficacious in the composite exposure with a small dose of another chemical (Arnold et al., 1996; Westin et al., 1998). Simultaneous treatments of *Bufo arenarum* embryos with Cd^{2+} and 17β-estradiol enhanced the lethality exerted by cadmium. The results indicate that estrogenic endocrine disruptors could have an adverse effect on amphibian embryos and enhance the toxic effect of Cd on amphibian embryos (Fridman et al., 2004). Sogawa and his colleagues investigated the effects of bisphenol A (BPA) on hepatic metallothionein (MT)-I mRNA expression and MT contents after Cd injection in experimental animals (Sogawa et al., 2001). They showed that BPA reduced the Cd-induced expression of MT-I mRNA and MT protein in the liver, resulting in increased damage to the liver. However, the administration of tamoxifen, an estrogen receptor antagonist, prevented these effects, indicating the synergism was mediated by estrogen receptor.

Comparing with these previous reports, the pretreatment of ICI 182,780 could not prevent the mutual synergistic cytotoxic effect in the present study, which supports that the synergism observed in our study were not through estrogen receptor but from direct cytotoxic effects of the chemicals (Fig. 4). It has been suggested that estrogenic activity and cytotoxicity are not directly correlated (Vega-Lopez et al., 2007). They reported that PCBs concentration needed to obtain estrogenic effects was lower by one order of magnitude than that necessary to induce cytotoxicity. Typical endocrine disrupting chemicals (EDCs) disturb the receptor-dependent effects of natural hormones. Although estrogenic activities of EDCs have been mainly investigated, EDCs include chemicals that potentially affect the functions of other nuclear receptors as well. Since natural ligands of nuclear receptors, such as pregesterone, glucocorticoids, and retinoic acids, exert direct effects on T cells to suppress Th1 development and enhance Th2 development (Iwata et al., 2003). BPA increased vulnerability (decrease of cell viability and differentiation, and increase of apoptotic cell death) of undifferentiated PC12 cells and cortical neuronal cells isolated from gestational 18-day rat embryos in a concentration-dependent manner (more than 50 μM). The ER antagonists, ICI 182,780, and tamoxifen, did not block these effects. The cell vulnerability against BPA was not significantly different in the PC12 cells overexpressing ER-α and ER-β compared with PC12 cells expressing vector alone (Lee et al., 2007). These results support that the synergistic effect of $HgCl_2$ and NP of the present study may also generate the toxicity in an estrogen receptor independent manner.

From the results presented above, NP and $HgCl_2$ caused oxidative stress and they decreased intracellular GSH (Fig. 5). The cytotoxicity observed upon the simultaneous treatment of NP and $HgCl_2$ was completely blocked by pretreatment of ROS scavenger, suggesting that it is mediated by ROS generation (Fig. 6). The uptake of mercury chloride showed that kidney and liver were accumulated with the highest levels of mercury compared to other organs in animal studies (Hussain et al., 1999). A dose-dependent increase of antioxidant enzymes was occurred in this liver. The increase in the enzyme activities was also well correlated with the accumulation of mercury in liver. Mercury exposure in experimental animals has been previously demonstrated to induce lipid peroxidation detected by increased thiobarbituric acid-reactive substances (TBARS) in liver, kidney, brain, and other tissues (Huang et al., 1996). Addition of $HgCl_2$ to isolated hepatocytes caused a rapid increase in ROS formation and a decline in mitochondrial membrane potential (Pourahmad et al., 2001). These cells eventually developed lipid peroxidation and cell lysis. Also NP has been reported as a compound of strong oxidative stress damage. From the cytotoxicity evaluation of environmental pollutants, NP has been shown to be a strong inducer of GSH depletion among other endocrine disruptors (Toyo'oka et al., 2001). NP-induced oxidative stress and toxicity has been recently demonstrated in testicular Sertoli cells (Gong and Han, 2006). In the study, Sertoli cells exposed to NP for 24 h exhibited cell death and growth inhibition which was associated with intracellular accumulation of ROS. In our experiment, we used each of NP and $HgCl_2$ at concentration that induces a marginal decrease of reduced cellular GSH level when applied separately. On the other hand, their co-treatment significantly decreased intracellular levels of reduced GSH. The decrease of intracellular reduced GSH levels was highly correlated with the decrease of their cell viabilities.

In summary of our study, the cytotoxicity of $HgCl_2$ and NP on HepG2 cells are mediated by ROS generation which is estrogen receptor independent. In our real life, it is generally granted that we are continuously and often simultaneously exposed to many different kinds of environmental pollutants. The present study suggests a possible mutual synergistic toxicity between toxic pollutants that may be encountered in actual living organisms.

Conflict of interest

None.

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