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Experimental study on the estrogen-like effect of mercuric chloride

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Abstract Although mercuric chloride has toxicity on reproductive system, it is uncertain if such toxicity is induced by estrogen-like effect. To study whether mercuric chloride has the estrogen-like effect and its relevant mechanism, proliferation assay of MCF-7 human breast cancer cells, uterotrophic assay, peroxidase activity assay and estrogen receptor competitive binding assay were conducted to screen the estrogen-like effect of mercuric chloride. The MCF-7 cells proliferated in the stimulation of mercuric chloride and got to the peak at 10^{-7} mol/l concentration. And this proliferation could be completely blocked by estrogenic antagonist ICI182.780. In addition, mercuric chloride could increase the weight of uterus of ovariectomized SD rats and the peroxidase activity of uterus complying with dose-effect relationship. However, mercuric chloride could not affect the binding of estradiol (E_2) to estrogen receptor (ER). So mercuric chloride exhibits the estrogen-like effect through binding and activating ER rather than bind to ER by competing with E_2.

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Introduction

In recent years, growing studies have shown that some chemicals in environment can disrupt the synthesis, secretion, transportation, combination, metabolism or elimination of the natural hormones in the body, which called environmental endocrine disruptors (EEDs). EEDs have been noticed widely in toxicological studies (Colborn et al. 1993; McLachlan and Korach 1995; Oberdorster and Cheek 2001; Orchinik and Propper 2005). A series of adverse effects such as tumors dependent of hormone, dysfunction of immune system and nerve system, particularly reproductive and developmental systems damages are induced by EEDs through simulating or antagonizing natural hormones (Keith 1997). Among them, environmental estrogens (EEs) are important substances which have similar functions of endogenic estrogens.

A variety of EEs are ubiquitously distributed in environment, including the dioxins, dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenylethylene (DDE), hexachlorocyclohexane, polychlorinated biphenyls (PCBs), and alkylphenols and their derivatives, which can be found in pesticides, pet, detergents, birth control pills, plastics (PVCs), PCBs,

oil refining, auto and truck exhaust, cigarette smoke, coal burning power plant emissions (Watson et al. 1999; Aravindakshan et al. 2004; Wozniak et al. 2005). They have shown manifold influences on the reproduction and development, tumor formation, nerve system, immune system of human and animals through food chain or contact directly (Bevan et al. 2003; Althuis et al. 2003; Sthoeger et al. 2003).

Some toxic metals like lead, cadmium and organic tin have been found to have definite estrogen-like effect (Wide and Wide 1980; Garcia et al. 1994). However, there are still other metals in environment, such as mercury, manganese and chromium, to which we are not certain whether they have the estrogen-like effect. Although mercury can prohibit the forming of sperm and reduce the content of some endogenous hormone, the existing data is insufficient for us to confirm whether these damages were caused by the estrogen-like effect of mercury (Xiaoyu et al. 1993; Rao and Sharma 2001). The purpose of this study is to detect whether mercuric chloride has the estrogen-like effect through a series of assays, including MCF-7 human breast cancer cells proliferation assay, uterotrophic assay in ovariectomized SD rats, peroxidase activity assay and ER competitive binding assay, therefore to provide experimental evidence for further screening its estrogen-like effect.

Materials and methods

Materials: Adult female SD rats whose bodies' weights ranged 210–230 g were provided by Experimental Animal Center of Henan province (the number was 410117). The use of the animals was reviewed and approved by the Henan Experiment Animal Committee. All animal experiments were performed on freely moving female SD rats. The rats were caged with free access to food and water, and maintained in a room temperature of 20 ± 2°C with a 12 h light–dark cycle. All experiments were conducted according to the guidelines of the International Association for the Study of Pain (Zimmermann 1983) and efforts were made to minimize the animal sufferings.

Cell strain: MCF-7 human breast cancer cell strain was purchased from Cell Biology Research Institute of Chinese Science Academy. Estrogen was purchased from Sigma Company. Calf serum was from TBD bio-tech development center. Fetal bovine serum treated by dextran-charcoal was purchased from Hyclone Company and RPMI-1640 from Gibco Company. ICI182.780 was purchased from DOCRIS Company. 2, 4, 6, 7-^{3}H E_2 was purchased from AMERSHAM.

Proliferation assay of MCF-7 human breast cancer cells

MCF-7 cells were grown in RPMI-1640 growth medium supplemented with 10% calf bovine serum and 30 U/100 ml insulin. After the cells were incubated for 3 days, the medium was removed and cells were washed by PBS four times, then cloned MCF-7 cells trypsinized with 0.25% trypsin were grown in experimental medium (phenol red-free RPMI-1640 supplemented with 5% charcoal–dextran-treated fetal bovine serum) and placed into a 96-well cell culture plate at initial concentrations of 2000 cells. Estrodiol was dissolved in ethanol, diluted with phenol red-free RPMI-1640 at different concentrations and the final concentration of ethanol was lower than 0.1%. After 24 h incubation in experimental medium, the different concentration of mercuric chloride and estrodiol were added into the wells respectively (Soto et al. 1995; Martin et al. 2003a). The culture was continued for 6 days, and the cell number was estimated by performing the MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay (Jieyun and Qiang Xu 2003; Ju et al. 2004). A total of 25 μl sterile solution of MTT were added into the wells and incubated for 4 h. The supernatant was substituted by 150 μl of dimethyl sulfoxide (DMSO) and the absorbance of the dissolved formazan measured at 490 nm in an enzyme-linked immunosorbent assay plate reader (DG5031, Donghua Instru. Co.). Proliferation index (PI) was expressed as percentages of treated cultrues with negative control, as well as Relative proliferation potency (RPP) was expressed as percentages of maximal treated cultures with maximal estradiol cultures.

Uterotrophic assay in ovariectomized SD rat

Female adult SD rats were ovariectomized 16 days before the start of the experiment. The animals were acclimatized for 2 days before being dosed. At the

beginning of assay, the animals were weighed, randomized to 5 groups (8 rats per group) and administered one of the followings for 3 consecutive days. All rats were dosed by intraperitoneal injection with solutions (2.0 ml/kg body weight) of mercuric chloride or 17β-estradiol or distilled water. Different concentrations of mercuric chloride were prepared to be reserved liquid by distilled H_2O and diluted to corresponding using liquids before experiment. Estrodiol was dissolved by peanut oil and diluted with distilled H_2O before experiment. Group 1: negative control (distilled water); Group 2: low dose mercuric chloride (0.04 mg/kg); Group 3: medium dose mercuric chloride (0.20 mg/kg); Group 4: high dose mercuric chloride (1.00 mg/kg); Group 5: positive control (17β-estradiol 0.1 mg/kg). Twenty-four hours after being given the last dose, rats were weighed and sacrificed by cervical vertebrate luxation. The uteruses were excised quickly, trimmed free of connective tissue and fat, pierced and blotted to remove excessive fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. The uteruses were then weighed (wet weight). The organ coefficient of uterus was expressed as: wet weight of uterus / body weight (Diel et al. 2000; Lijun et al. 2001).

Peroxidase (POD) activity assay

The uteruses were cut into fragments and homogenized in ice bath with appropriate pre-cooled 5% $CaCl_2$. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C, then the supernatant was preserved as enzyme extract. Protein concentration of enzyme extract was determined according to Bradford (1976). The POD activities were determined photometricallly by adopting a light modification method described by Rodriguez and Tames (1982). The assay mixture contained 1.0 ml of 0.3% guaiacol, 0.5 ml of 26 mM H_2O_2 and 0.2 ml enzyme extract as substrate. The mixture was incubated for 15 min at 20°C, and finally 2 ml acetone was added to cuvette. Optical density (OD) at 475 nm was measured using a spectrophotometer (722S, SOIEC). POD activities were expressed as milligram of guaiacol oxide per min per milligram of protein, according to the method of standard [Ag^+] curve (Yan 2000).

ER competitive binding assay

The validated ER competitive-binding assay was utilized to determine the ER affinity of mercuric chloride. Uteruses from ovariectomized SD rats were the ER source for the competitive binding assay. The detailed procedures of estrogen receptor preparation and Estrogen receptor (ER) competitive-binding assay were performed according to Blair et al. (2000). Radioactivity was measured on a FJ-2101G Liquid scintillation counter(Xi'An, China). The assay included a zero tube (no competitor added; represented total binding of ^3H-E_2)and an E_2 standard curve (8×10^{-8}, 4×10^{-8}, 2×10^{-8}, 1×10^{-8} and 0.5×10^{-8} mol/l concentrations) for quality control purpose. The 8×10^{-8} mol/l E_2 tube contained a 100-fold molar excess of radioinert E_2 compared to ^3H-E_2 and thus represented non-specific binding (NSB). Radioactivity counts of the NSB tubes were subtracted from all tubes prior to calculation of percent ^3H-E_2 bound. Data were plotted as percent ^3H-E_2 specific bound versus logarithmic molar concentration (Baozhang et al. 2000).

Statistic methods

One-factor analysis of variance was used to analyze all the data which were represented with $\bar{x} \pm s$. The level of significance test was $\alpha = 0.05$. All statistical analyses were conducted with SAS 8.1 statistics software.

Results

Proliferation assay of MCF-7 human breast cancer cell

The proliferation effect of estradiol

Figure 1 presented that 10^{-12} mol/l E_2 could induce MCF-7 to proliferate and the proliferating index was 1.28 fold as that of negative control; 10^{-9} mol/l E_2 had the greatest effect on MCF-7 and the proliferating index was 4.48 fold. The differences in absorption values between each concentration of estradiol group and negative control had statistical significance ($P < 0.05$). Consequently, 10^{-9} mol/l estradiol was used as the concentration of the positive control when

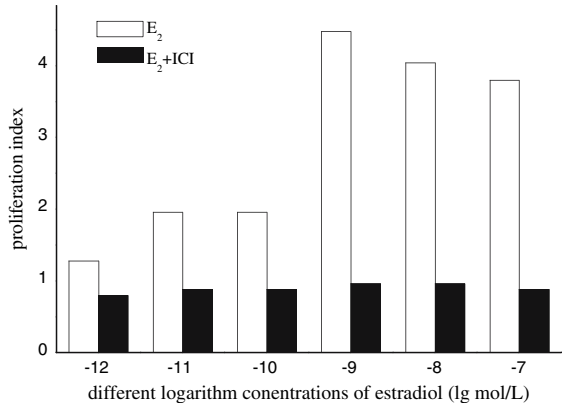

Fig. 1 The proliferation effect of different concentrations of estradiol to MCF-7 cells and the antagonistic effect of estrogenic antagonist ICI182.780

detecting the proliferation effect of mercuric chloride to MCF-7 cells. In this assay, the proliferation effect of estradiol to MCF-7 cells could be totally blocked up by 10^{-6} mol/l estrogenic antagonist ICI182.780.

The proliferation effect of mercuric chloride

Figure 2 indicated that the proliferation effect of mercuric chloride to MCF-7 was similar to estradiol and showed dose-effect relationship. The differences in absorption values between 10^{-5} and 10^{-9} mol/l of mercuric chloride group and negative control had statistical significance ($P < 0.05$). Among them the index of 10^{-7} mol/l group was as much as 3.08 fold compared to negative control. The relative proliferation index of 10^{-7} mol/l mercuric chloride was 59.8%

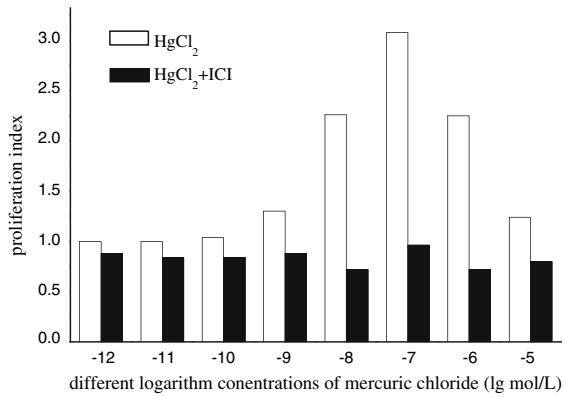

Fig. 2 The proliferation effect of different concentrations of mercuric chloride to MCF-7 cells and the antagonistic effect of estrogen antagonist ICI182.780

of that of estradiol and the relative proliferation potency was 0.01.

The uterus weight of ovariectomized SD rats and the organ coefficient of uterus

The average wet weight of uterus and the organ coefficient of uterus of positive control were obviously higher than those of the negative control ($P < 0.05$). Mercuric chloride induced significant increase in uterine weight in the maxium dose group (120% negative control; $P < 0.05$). E_2 induced even greater increasing of uterine weight (249%; $P < 0.05$) (see Table 1).

Peroxidase activity of uterus in ovariectomized SD rats

The peroxidase activity of uterus of positive control was apparently higher than that of the negative control ($P < 0.05$). The peroxidase activity of uterus increased with the increasing concentration of mercuric chloride. Moreover, there was significant difference between the high concentration of mercuric chloride group and negative control ($P < 0.05$) (see Table 2).

ER competitive binding assay

Scatchard diagram (see Fig. 3) was made with saturation analysis and linear regression equation was got in form of $y = -0.0218x + 0.0794$. The biggest capacity of estrogen receptor (Bmax) was 3.642 fmol/mg.prot. and the connection dissociation constant (Kd) value was 2.29×10^{-10} mol/l. When the final concentration of ^{3}H-E_2 was 8 nmol/l, the biggest capacity of ER was 3.630 fmol/mg.prot which was 99.6% of theoretical capacity. So the final concentration of ^{3}H-E_2 of 10 nmol/l was applied in one point saturation analysis.

It was presented in Fig. 4 that the binding of ^{3}H-E_2 with ER extracted from the uteruses of ovariectomized SD rats didn't decrease obviously with the increasing concentration of mercuric chloride. The difference between the cpm counting of the subject of each concentration and that of negative control hadn't statistical significance ($P > 0.05$).

In all, the relative proliferation index of 10^{-7} mol/l mercuric chloride was 59.8% of that of estradiol

Table 1 The uterus wet weight of ovariectomized SD rats and the organ coefficient of uterus in the group of mercuric chloride ($\bar{x} \pm s$)

Group	Rats number (n)	Average wet weight of uterus (g)	Organ coefficient of uterus ($\times 10^{-3}$)
Negative control	8	0.1108 ± 0.0195	0.3913 ± 0.0442
0.04 mg/kg$HgCl_2$	8	0.1011 ± 0.0100	0.3470 ± 0.0429
0.20 mg/kg$HgCl_2$	8	0.1266 ± 0.0142	0.4405 ± 0.0551
1.00 mg/kg$HgCl_2$	8	0.1339 ± 0.0190*	0.4743 ± 0.0627*
0.10 mg/kgE_2	8	0.2764 ± 0.0436*	0.9887 ± 0.1593*

Note: analysis of variance of average wet weight of uterus $F = 67.93$, $P < 0.0001$; analysis of variance of organ coefficient of uterus $F = 78.54$, $P < 0.0001$; * compared with negative control $P < 0.05$

Table 2 Peroxidase activity of uterus in ovariectomized SD rats in the group of mercuric chloride ($\bar{x} \pm s$)

Group	Rats number (n)	POD activity [mg/(mg.prot.min)]
Negative control	8	1.1120 ± 0.1269
0.04 mg/kg$HgCl_2$	8	1.0434 ± 0.1354
0.20 mg/kg$HgCl_2$	8	1.1630 ± 0.1070
1.00 mg/kg$HgCl_2$	8	1.3178 ± 0.1996*
0.10 mg/kgE_2	8	2.0319 ± 0.1913*

Note: $F = 53.16$, $P < 0.001$; *compared with negative control $P < 0.05$

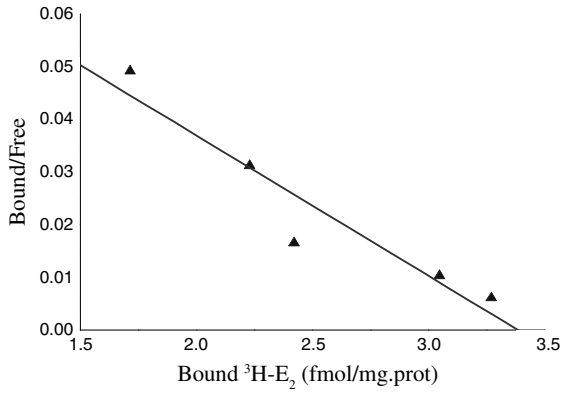

Fig. 3 Scatchard plot of saturation binding of ^{3}H-E_2

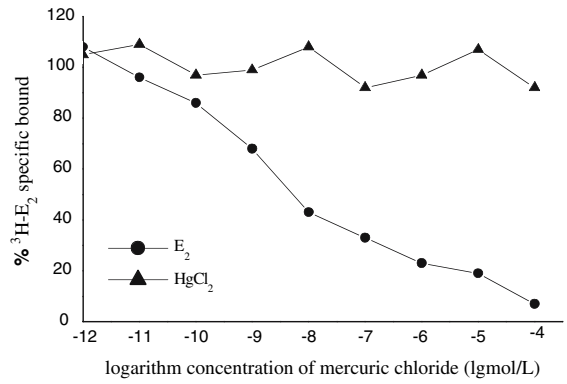

Fig. 4 The estrogen receptor binding curves

in vitro. The average wet weight of uterus , the organ coefficient of uterus and the peroxidase activity of uterus increased complying with dose-effect relationship in vivo.

Discussion

It was previously showed that the metal cadmium mimic the biological functions of estradiol in breast cancer cells by activating the ER (Garcia et al. 1994; Stoica et al. 2000). The purpose of this study was to determine whether mercury mimic the effects of estrogens. The present study demonstrated that the mercuric chloride had a significant effect on ER activity. Similar to estradiol, mercuric chloride made MCF-7 cells proliferate, induced the uterus of ovariectomized SD rats grow and enhanced peroxidase activity. Antiestrogen could block the ability of mercuric chloride to mimic the functions of estradiol

which suggested that its effects were mediated by ER. This result was consistent with that of Martin et al. (2003b). In their researches, it has been revealed that divalent cations, including cadmium, copper, vanadium, cobalt, mercury, and tin, may all mimic to some extent the effect of estradiol on cells expressing ERs. Martin and colleagues have extensively studied the estrogen-like effect of cadmium in cell cultures and in mouse model systems.

In proteins, metals serve several functions, including participation in catalytic reactions and stabilization of protein structure. Through their interactions with amino acids, metals can promote local folding, as in the case of the zinc fingers of the ER, or assembly of different regions of the protein into one domain (Hughes 1981; Predki and Sarkar 1992; Rodgers et al. 2001; Hartwig et al. 2002). The divalent metal ions copper, cobalt, nickel, lead, mercury, tin, and chromium, appear to activate ER through a mechanism involving cysteines C381 and C447, histidine H524, and the negatively charged amino acids glutamic acid E523 and aspartic acid D538. It is uncertain whether those amino acids form the metal-binding site, possibly through a direct interaction with the metal or an indirect interaction with water, or whether some of these amino acids recruit the metal to the binding site. The ability of metals to bind with high affinity and activate ER suggests that at environmentally relevant doses these compounds may pose a risk for endocrine-related diseases. In fact, exposure to metals is associated with endocrine imbalances and significant reproductive toxicity (Uzych 1985). In women, exposure to lead and mercury is linked to infertility, miscarriage, preeclampsia, pregnancy hypertension, premature delivery, and an increase in menstrual cycle disturbances. In experimental animals, exposure to these metals results in an inhibition of menstruation, ovulation, and follicular growth; a delay in vaginal opening; and an decrease in pregnancy (Barlow and Sullivan 1981; Levin and Goldberg 2000).

Mercury exists in different forms, including elemental mercury, inorganic mercury and organic mercury compounds. They have some properties in common but differ in metabolism and toxicity. Biotransformation takes place in the body, particularly the transformation of metallic mercury vapor to mercuric compounds, which means that some of the effects of inorganic mercury could also be expected after exposure to metallic mercury vapor (WHO 1991). There are a number of possible pathways for non-occupational exposure to inorganic forms of mercury. These include (1) eating fish or wild game near the top of the food-chain that have accumulated mercury in their tissues; (2) playing on contaminated surface soils; (3) playing with liquid mercury from broken electrical switches, thermometers, etc.; (4) bringing any liquid mercury or broken mercury device into the home, where vapors might build up in indoor air. Exposure from ambient air and drinking water is usually minor. Most human exposure to biologically significant amounts of elemental mercury occurs in the workplace. Workers in the chloralkali, electrical light bulb manufacturing, thermometer, and other industries where elemental mercury is utilized are exposed to levels much higher than the general population. Occupational mercury exposures generally occur when workers inhale elemental mercury vapors. Some dermal absorption may occur from skin contact with contaminated air, but the extent is low (less than 3% of the inhaled dose). Gold mining operations in Peru, Brazil, the Philippines, and less industrialized nations result in exposure for both miners and their families alike. Elemental mercury has the ability to readily cross the placental barrier. Thus, the developing fetus can be exposed to mercury from the pregnant woman's body through the placenta. Infants may also be exposed to mercury from a nursing mother's milk (ATSDR 2003).

Although the precise way in which mercury showed estrogen-like effect remains to be determined, its ability to function as potent estrogens suggests that mercury may be an important endocrine disrupters. Because exposure to such metal is widespread, the elucidation of its roles in the etiology and development of hormone-related diseases may have significant implications in risk reduction and disease prevention.

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