# ESTROGENICITY IN CRUDE AND FRACTIONATED EXTRACTS OF MEDICAL WASTE INCINERATOR ASHES

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## Introduction

A various types of organohalogen compounds are formed as by-products through thermal process of waste combustion [1,2]. Thus, to know potential toxicity of incinerator emission is essential issue for occupational health management. The study on the workers who handle fly ashes at highly contaminated incinerator has revealed that their plasma dioxin concentrations were extremely high [3]. It also has been suggested that occupational contamination with chlorophenols and heavy metals can be occurred at an older incineration plant without relevant safety measures [4].

Among the compounds that can be found in emission, some are known as environmental estrogens. Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/DFs), which are the well-known by-products, have antiestrogenic effects *in vitro* and *in vivo* [5,6]. On the other hand, some polychlorinated biphenyls (PCBs) congeners exhibit estrogenic activity [7,8]. Moreover, hexachlorobenzene and pentachlorophenol that are precursors of dioxins in an incineration plant also alter endocrine functions in experimental animals or in molecular levels [9,10]. Therefore, hazard and risk assessment of emissions from an incinerator should take the interactions of chemicals into account, not mere quantitative measurement of compounds, in order to estimate overall estrogenic effect. For this purpose, bioassay method using either cell-lines or laboratory animals has been developed and utilized [11].

In this study, estrogenicity of ashes collected from a medical waste incinerator was assessed by an *in vitro* estrogen assay method using crude and fractionated extracts of the samples prepared for dioxin analysis.

## Materials and Methods

*Samples, Extractions, Fractionations and Dioxin analysis*

Bottom ashes were collected from a medical waste incinerator located in Yokohama, Japan once a day for five consecutive days. The samples were well homogenized, weighed out, and extracted with toluene in a Soxhlet apparatus. The crude extracts were treated with sulfuric acid and further clean-up procedure was performed using silicagel, alumina, and silica-mixed activated charcoal column according to the methods described in elsewhere [12]. The procedure is shown in figure 1. PCDDs/DFs and coplanar PCBs were determined by high-resolution gas chromatograph (HP5890II, Hewlett Packard, USA) / high-resolution mass spectrometry (SX102A, JEOL, Japan) with selected ion monitoring. The condition of the analysis was based on a method which is described in a previous report [12]. Crude extract (CE), second fraction of silicagel column chromatography (SL), first fraction of alumina column chromatography (AL), and first and second fractions of active charcoal

chromatography (AC, and DX) were used for estrogenicity test. The solvents of all the extracts were replaced with cell-culture grade dimethyl sulfoxide (DMSO) for the assay.

*Detection of Estrogenic activity*

Stably transfected ME2L cells, which is MCF-7 cells (ATCC No. HTB-22) with pGL2-promoter plasmid that contained two copies of xenopus vitellogenin A2 estrogen responsive element genes [13] in its promoter area were established in our laboratory. The ME2L cells were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum, sodium pyruvate, L-glutamine, MEM non-essential amino acids, insulin, and penicillin-streptomycin- amphotericin B in an incubator maintained with 5% $CO_2$, 95% air and 100% humidity at 37°C. The medium was replaced with phenol red free DMEM with 5% dextran-coated charcoal treated FBS (PRF-DMEM/DCC-FBS) at 50% of confluence prior to the assay. Two days after the replacement, the cells were seeded at $4x10^4$ cells per well in 96-well plates and maintained in 100µl of PRF-DMEM/DCC-FBS at 37°C. After 12 hours, 100µl of the same medium that contained the tested extracts in various concentrations was added. DMSO, estradiol (E2) and 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) were used as control, reference and inhibitor, respectively. After further 24 hours of incubation, medium was removed and the cells were washed by phosphate-buffered saline twice, and harvested with 50µl of reporter lysis buffer (Promega). The luciferase activity was determined with luciferase assay system and TD-20/20 luminometer (Promega). Protein concentration was measured with BCA protein assay reagent (Pierce) using bovine serum albumin as standard. The luciferase activity was adjusted by protein concentration. The results obtained from the extracts, E2 or TCDD were divided by the results from control in order to determine fold induction. The data shown are average of three replicates of the assay.

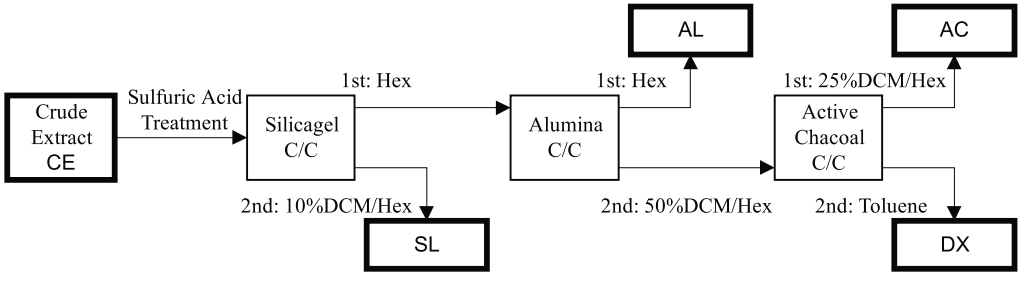


**Figure 1.** Extraction and fractionation procedure of medical waste incinerator ashes collected in Japan. CE, SL, AL, AC and DX show the extracts used in this study. (C/C: column chromatography, Hex: Hexane, DCM: dichloromethane)

**Results and Discussion**

Luciferase activity induced by E2 in ME2L cells was dose-dependent manner (Table 1). 500pM of E2 induced maximum luciferase activity, with a 6.2 fold in comparison with control. The induction decreased by 0.8 fold with 1000pM of TCDD.

Fold inductions in luciferase activity by the extracts and dioxin concentration in ashes are shown in table 2. The levels of induction varied among the samples and the extracts. Crude extracts in sample 1, 3, and 5 exhibited relatively high luciferase induction level, which was beyond the activity by 15pM of

E2, despite the extracts contain various amount of dioxins which have antiestrogenic effect. The induction levels of SL fractions in the same samples were lower than those of CE, therefore some chemicals that have estrogenic effect may be degraded during sulfuric acid treatment or distributed to the acid layer. However, luciferase levels of SL fractions in sample 1 and 3 were still almost twice as high as that of control. This data suggest that the ashes contain certain amounts of estrogenic compounds, and these chemicals can be fractionated in the second fraction of silicagel column chromatography. Since DDTs, hexachlorocyclohexane and chlordanes are known to be eluted in the SL fraction, further investigation is required to detect the responsible chemicals and to estimate magnitude of estrogenic effect.

In contrast with the above results, crude extract of sample 4 decreased luciferase induction, on the other hand, SL fraction in the same ashes displayed 1.9 times higher luciferase induction than control. It implies that antiestrogenic activity was dominant in the ashes, however estrogenic effect emerged in SL fraction due to disappearance of antiestrogenic compounds by sulfuric acid treatment or fractionation.

Decreases in luciferase induction were observed in most of AL, AC and DX fractions in each sample. Di-*ortho* PCBs, mono-*ortho* PCBs, and PCDD/DFs can be fractionated in AL, AC, DX fractions, respectively, thus antiestrogenic effect of dioxins may be dominant ant estrogenic compounds may be low in quantity in these fractions. However, each step of ERE-derived luciferase assay, which is ligand-estrogen receptor complex formation and its binding to ERE, transcription, translation, protein transportation or enzyme reaction, may also be inhibited by unknown component in the fractions. Therefore it is difficult to estimate antiestrogenic effect of dioxins in complex mixture only with this assay result. This could be one of the obstacles in utilisation of bioassay.

This study suggests that ashes from medical waste incinerator contained both estrogenic and antiestrogenic chemicals. These compounds could alter endocrine system and eventually cause diseases in humans. Emission control from incinerator is usually focused only on dioxins using toxic equivalency factor approach. In occupational health management, however, estrogenicity of ashes should be taken into account. Further studies on estrogenicity detection in ashes, on estimation and identification of responsible chemicals, and on bioavailability and metabolism of the chemicals in humans are required.

**References**

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**Table 1.** Fold induction of luciferase by estradiol (E2) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in ME2L cells.

| Chemicals | E2 | | | | | TCDD |
|---|---|---|---|---|---|---|
| Concentration (pM) | 4 | 16 | 62.5 | 250 | 500 | 1000 |
| Fold Induction | 1.0 | 2.4 | 3.9 | 3.4 | 6.2 | 0.8 |

**Table 2.** Fold induction of luciferase by crude and fractionated extracts, and dioxin concentrations of crude extract in the estrogenicity assay condition, in medical waste incinerator bottom ashes.

| | Sample Number | | | | |
|---|---|---|---|---|---|
| Fraction | 1 | 2 | 3 | 4 | 5 |
| CE | 4.1 | 1.4 | 3.0 | 0.4 | 2.4 |
| SL | 1.8 | 0.7 | 1.9 | 1.9 | 1.1 |
| AL | 1.2 | 0.4 | 0.8 | 0.9 | 0.6 |
| AC | 0.8 | 1.1 | 0.3 | 0.5 | 0.9 |
| DX | 0.7 | 0.7 | 0.0 | 1.2 | 1.1 |
| Dioxin concentration of CE (pM / well-tested) | 5.95 | 5.55 | 0.67 | 0.14 | 12.10 |