THE ESTROGENIC EFFECTS OF PHENOLIC COMPOUNDS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN MCF-7 CELLS AND UTERINE GLUTATHIONE PEROXIDASE IN RATS

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Introduction

Some phenolic compounds are used in a number of commercial products and have been reported to be weakly estrogenic in previous studies[1,2]. Bisphenol A, used as coating material for food can and dental sealants, is relatively more estrogenic than other phenolic compounds, such as 4-nonylphenol, 4-octylphenol and 4-propylphenol[3,4]. Their estrogenic activities are mainly dependent on their binding affinity for the estrogen receptors in vitro and in vivo. Although there are various methods shown to be useful approaches for identifying endocrine disruptors through direct interaction with the estrogen receptors[5], it is required that the method protocol selected be capable of detecting chemicals with a broad range of estrogenic activity. In this study, we tested phenolic compounds such as bisphenol A, 4-nonylphenol, 4-octylphenol and 4-propylphenol by using glucose-6-phosphate dehydrogenase (G6PD) in estrogen sensitive human breast cancer cells (MCF-7 cells) and glutathione peroxidase (GPx) in female immature Sprague-Dawley (SD) rats. This study was designed to investigate whether G6PD and GPx assay could be useful screening methods for endocrine disruptors.

Methods and Materials

Chemicals. 17β-Estradiol and bisphenol A were purchased from Sigma Chemical Co. (MO, USA). 4-Nonylphenol, 4-octylphenol and 4-propylphenol were obtained from Aldrich Chemical Co. (WI, USA).

Animals. For determination of GPx activity, female SD rats were obtained at the age of 19-day from Korea Food & Drug Administration (Seoul, Korea).

Cell Culture and Treatment[6]. MCF-7 cells for G6PD assay were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS plus penicillin/streptomycin, 4 mM L-glutamine, and 0.6 μg/mL insulin. Cells were harvested at 37℃ in a humidified atmosphere containing 5% CO_2 in air and were passaged every 3 days using 0.25% trypsin-1mM EDTA in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS). In order to remove cells of endogenous steroids, the medium was replaced by DMEM containing 2.5% FCS/CDS (charchoal dextran-stripped, treated fetal calf serum). Two days later, cells were trypsinized, and the cell suspension was plated (2×10^4 cells/well) on 48-well plate in 2.5% FCS/CDS medium, without insulin. Two days after plating to the bottom of the well, the media were removed and replaced by insulin-free, 2.5% FCS/CDS medium containing phenolic compounds in ethanolic solution with a final concentration of less than 0.1%. The control medium contained an equivalent volume of ethanol. The media containing MCF-7 cells and phenolic compounds were incubated for 6 hr, and

then G6PD assay was performed.

***G6PD Assay*[7]**. G6PD activity in cultured MCF-7 cells was measured by quantitative cytochemical determination of produced NADPH by using PMS-NT procedure. The media were removed after 6 hr incubation and the cells were rinsed. G6PD activity was determined by incubation of the cells in a medium containing 1.5 mM G6P, 1 mM NADP, and 7.5 mM neotetrazolium (NT), in the presence of 0.1mM phenazine methosulfate (PMS) or in the absence of PMS for 1.5 hr at 37℃. To extract formazan generated, DMSO was added to the colored well. The amount of formazan was measured at 540 nm by using UV-VIS spectrophotometer (Milton Roy, USA). The difference of formazan amount between in the presence and the absence of PMS was calculated to determine the direct synthesis of formazan by G6PD.

***Uterine GPx Assay*[8]**. Uterine GPx activity was assayed in 19-day female rats. 17β-Estradiol (0.1, 0.3, and 1 μg/kg), bisphenol A (20, 100, and 500 mg/kg), 4-nonylphenol (500 mg/kg), and 4-octylphenol (500 mg/kg) were treated for 3 consecutive days by subcutaneous injection. The next day of the last treatment, immature rats were killed by cervical dislocation. Endometrial specimens were homogenized in 0.25 M sucrose containting 150 mM KCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5), and the homogenate was centrifuged at 800 g for 20 min at 4℃. The supernatant was centrifuged at 10,000g for 20 min at 4℃. The supernatant was recentrifuged at 100,000 g for 2 hr at 4℃and the subsequent supernatant was assayed for GPx activity. GPx activity was determined with H_2O_2 as a substrate and by measuring the absorbance of NADPH at 340 nm.

Statistical Analysis. ANOVA was determined by using SPSS. Differences from control values in all cases were assessed statistically using Duncun's multiple-range test. The level of statistical significance was set at $P < 0.05$.

Results and Discussion

G6PD Assay. In order to determine the pertinent incubation time in our laboratory, MCF-7 cells were incubated with 17β-estradiol (10^{-11} ~ 10^{-6} M) for various periods (1 hr, 3 hr, 6 hr, and 24 hr). The G6PD activity was dramatically increased by treatment with 17β-Estradiol for 6 hr or 24 hr above the concentration of 1×10^{-7} M when compared with the control level. We chose the 6 hr as incubation time for G6PD assay. Above the concentration of 1×10^{-9} M, bisphenol A significantly increased the G6PD activity in a concentration-dependent manner, compared with control. 4-Nonylphenol (over the concentration of 1×10^{-9} M) also enhanced the G6PD activity about 1.8 times of control. 4-Octylphenol produced weaker effects on G6PD than 4-nonylphenol did, and showed the tendency of increasing the G6PD activity. 4-Propylphenol did not affect the G6PD activity and just induced cytotoxic effects at the concentration of 1×10^{-4} M (data not shown). This result shows that bisphenol A and 4–nonylphenol have the enhancing effect of G6PD activity in MCF-7 cell. G6PD, the rate-limiting enzyme of the pentose phosphate pathway, determines the production of NADPH by controlling the glucose metabolism via this pathway[9]. Therefore, G6PD is one of the principal sources of cytoplasmic NADPH, and its activity is influenced from the status of cell proliferation[10]. In this study, 17β-estradiol, bisphenol A and 4–nonylphenol stimulated highly G6PD activity. Previous studies presented that these estrogenic chemicals stimulated the proliferation of MCF-7 cells[11,12]. From these results, it could be thought that G6PD assay in MCF-7 cells is used as a new screening method for estrogenic compounds.

Uterine GPx Assay. GPx involves in the metabolism of lipid peroxides and plays a role in host defence mechanisms as an antioxidant enzyme[13]. In rats, hepatic GPx activity is higher in females,

and is higher in estrous phase than in the diestrous phase of the estrous cycle[14]. And endometrial GPx activity was significantly increased by exogenous estrogen and remarkably reduced by exogenous progesterone. In vivo GPx assay, 4-propylphenol was not tested because of lacking of increasing effect on G6PD activity in vitro. Both bisphenol A and 17β-estradiol significantly increased the wet uterus weights and dramatically enhanced uterine GPx activities in immature female rats in a dose-dependent manner. Treatment with 4-nonylphenol (500 mg/kg/day) increased significantly the uterine GPx activity and the wet uterus weights in immature female rats. 4-Octylphenol (500 mg/kg/day) also caused the significant increase in uterine GPx activity, but had no effect on the wet uterus weights. This finding indicates that the change in uterine GPx activities could be a more sensitive parameter than that of wet uterus weights in immature rats, and that uterine GPx assay may be a useful tool in vivo for screening estrogenic chemicals although the immature uterotrophic assay is the well-known method for endocrine disruptors.

These results suggest that G6PD assay in MCF-7 cells and uterine GPx assay in rats could be applicable to screening estrogenic activity of phenolic compounds.

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Table 1. The effect of 17β-estradiol, bisphenol A, 4-nonylphenol, 4-octylphenol on uterine wet weights and body weights.

Treatment (mg/kg/day)	Wet uterus weight (mg)	Terminal body weights (g)
Corn oil (control)	59 ±16	55.2 ±7.6
17β-estradiol (0.0001)	56 ±15	56.3 ±6.7
17β-estradiol (0.0003)	65 ±16	51.9 ±6.8
17β-estradiol (0.001)	108 ±18 **	51.0 ±7.2
Bisphenol A (20)	56 ± 9	52.6 ±4.1
Bisphenol A (100)	67 ±21	54.4 ±6.8
Bisphenol A (500)	95 ±18 **	55.0 ±5.1
Corn oil (control)	35 ± 6	50.9 ±3.7
4-Nonylphenol (500)	100 ±27 **	45.6 ±5.0
4-Octylphenol (500)	39 ± 6	44.5 ±4.2

**, P <0.01 (*versus* control value)

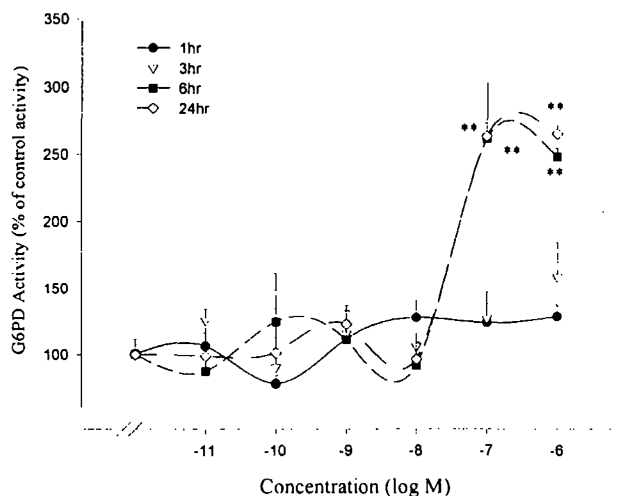

Fig. 1. The effect of 17β-estradiol on G6PD activity in MCF-7 cells. G6PD activity was measured in MCF-7 cells in the presence of 17β-estradiol for various incubation periods (•: 1 hr, ∇: 3 hr, ▪: 6 hr, ◊: 24 hr). Results are presented as % of control activity and expressed as the means ±S.D. of 4 wells. **, P <0.01 (*versus* control value)

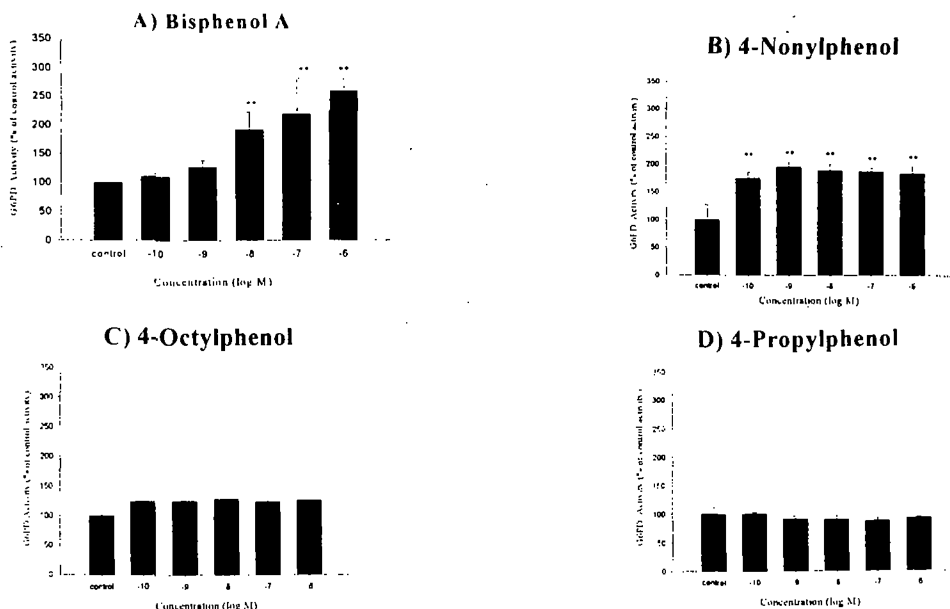

Fig. 2. Effects of phenolic compounds on G6PD activity in MCF-7 cells. G6PD activity was measured in MCF-7 cells in the presence of bisphenol A (A), 4-nonylphenol (B), 4-octylphenol (C) and 4-propylphenol (D). Results are presented as % of control activity, and are expressed as the means ±S.D. of 4 wells. **, P <0.01 (*versus* control value)

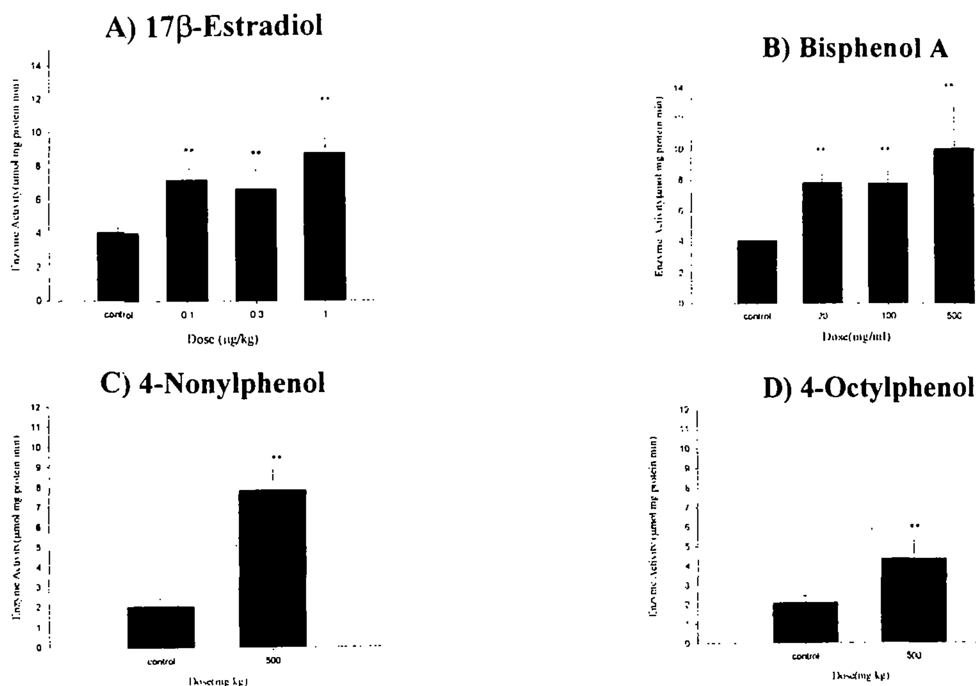

Fig 3. Uterine GPx activity in immature rats. Uterine GPx activity was determined from uterus of immature rats subcutaneously treated with 17β-estradiol (A), bisphenol A (B), 4-nonylphenol (C) and 4-octylphenol (D) for 3 consecutive days. Result are expressed as the means ±S.D. of 12 rats. **, P <0.01 (*versus* control value)