

## RESEARCH ARTICLE

# Tumorigenic Effects of Endocrine-disrupting Chemicals are Alleviated by Licorice (*Glycyrrhiza glabra*) Root Extract through Suppression of AhR Expression in Mammalian Cells

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## Abstract

Endocrine-disrupting chemicals (EDCs) have been reported to interfere with estrogen signaling. Exposure to these chemicals decreases the immune response and causes a wide range of diseases in animals and humans. Recently, many studies showed that licorice (*Glycyrrhiza glabra*) root extract (LRE) commonly called “gamcho” in Korea exhibits antioxidative, chemoprotective, and detoxifying properties. This study aimed to investigate the mechanism of action of LRE and to determine if and how LRE can alleviate the toxicity of EDCs. LRE was prepared by vacuum evaporation and freeze-drying after homogenization of licorice root powder that was soaked in 80% ethanol for 72 h. We used 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a representative EDC, which is known to induce tumors or cancers; MCF-7 breast cancer cells, used as a tumor model, were treated with TCDD and various concentrations of LRE (0, 50, 100, 200, 400 µg/mL) for 24, 48, and 72 h. As a result, TCDD stimulated MCF-7 cell proliferation, but LRE significantly inhibited TCDD-induced MCF-7 cell proliferation in a dose- and time-dependent manner. The expression of TCDD toxicity-related genes, i.e., aryl hydrocarbon receptor (AhR), AhR nuclear translocator, and cytochrome P450 1A1, was also down-regulated by LRE in a dose-dependent manner. Analysis of cell cycle distribution after treatment of MCF-7 cells with TCDD showed that LRE inhibited the proliferation of MCF-7 cells via G2/M phase arrest. Reverse transcription-polymerase chain reaction and Western blot analysis also revealed that LRE dose-dependently increased the expression of the tumor suppressor genes p53 and p27 and down-regulated the expression of cell cycle-related genes. These data suggest that LRE can mitigate the tumorigenic effects of TCDD in breast cancer cells by suppression of AhR expression and cell cycle arrest. Thus, LRE can be used as a potential toxicity-alleviating agent against EDC-mediated diseases.

**Keywords:** EDC - licorice root - cell cycle arrest - AhR - toxin - MCF-7 - breast cancer

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## Introduction

Endocrine-disrupting chemicals (EDCs) are natural or industrial compounds found in food and the environment that are capable of mimicking some of the effects of endogenous estrogens or interfering with estrogen signaling pathways by interacting with two estrogen receptors (ERs): ER $\alpha$  and ER $\beta$  (Pelekanou and Leclercq, 2011; Kerdivel et al., 2013). EDCs were first noted at the end of the 20th century. EDCs that target ER signaling can induce ER activity directly or indirectly through three signal pathways: directly through interactions with ERs, indirectly through transcription factors such as aryl hydrocarbon receptor (AhR), or through modulation of metabolic enzymes that are critical for normal estrogen synthesis and metabolism (Bidgoli et al., 2011; Shanle and Xu, 2011).

Endocrine disruption affects various body functions, depending on the pathway that is disrupted. It has been proposed that exposure to xenoestrogens lead to the increased prevalence of breast cancer (Abdel-Rahman et al., 2012; Kerdivel et al., 2013). TCDD, as a kind of EDC, has multiple endocrine activities and has been known to increase the incidence of breast cancer in regions contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) (Warner et al., 2002). Furthermore, treatment of breast cancer cells (MCF-7) with TCDD resulted in estrogen-like G0/G1 to S phase transition and mitogenic effects (Abdelrahim et al., 2002). The biological effects of TCDD are primarily mediated by binding to its intracellular receptor (AhR). Cytoplasmic AhR, upon TCDD binding, translocates to the nucleus. After dimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT), the complex binds to the

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dioxin-responsive element in the promoter region and regulates the expression of its target genes (Beischlag et al., 2008). Over the last two decades, endocrine disruption has gained more public, political, and scientific attention. It is interesting to note that endocrine disruptors have been around in hormone-dependent cancers for a long time (Cravedi et al., 2007; Bidgoli et al., 2012).

In recent years, special attention was given to the study of plants and their isolated compounds for the prevention of diseases and diverse pathological conditions. Licorice root (“gancao” in China and “gamcho” in Korea) is the most popular ingredient used in over 70% of Chinese medicines and has been used by human beings for at least 4000 years. This plant contains many flavonoids and pentacyclic triterpene saponins, including liquiritin, liquiritigenin, liquiritin apioside, glycyrrhizin, isoliquiritigenin, and glycyrrhizic acid (Kamei et al., 2003). Constituents of this plant have been reported to have a wide range of bioactivities, e.g., antimicrobial, anti-inflammatory, and cardiovascular protective activities (Fukai et al., 2003; Kang et al., 2005; Cheel et al., 2010). However, there is only limited information about if and how LRE can alleviate effects of endocrine disruptors.

Thus, this study aimed to investigate the mechanism of action of LRE and to determine if and how LRE can alleviate the toxicity of EDCs. We used TCDD as an EDC, which is known to induce tumors or cancers; MCF-7 breast cancer cells were used as tumorigenic model.

## Materials and Methods

### Chemicals and reagents

MCF-7 human breast cancer cells were obtained from Seoul National University (South Korea). TCDD was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum, phosphate-buffered saline, and Dulbecco’s modified Eagle’s medium were purchased from GIBCO BRL (Grand Island, NY, USA). Penicillin/streptomycin mix was purchased from Lonza (Walkersville, MD, USA). Cell counting kit-8 (CCK-8) reagent was purchased from Dojindo (Kumamoto, Japan). Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Ethidium bromide was purchased from Bio basic Inc. (South Korea) and Maxime PCR Premix (i-Taq) was purchased from iNtRON Biotechnology (Seoul, Korea). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, UK).

### Preparation of LRE

Dried licorice (*Glycyrrhiza glabra*) roots were freeze-dried and pulverized. The dried powder (500g) was then soaked in 80% ethanol for 24 h. The extracts were collected and the same process was repeated three times. The total extract was collected, filtered, and evaporated under reduced pressure. The end product was freeze-dried and the powdered extract was kept in a deep freezer (-70°C).

### Cell culture and treatment

MCF-7 human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with

10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mg/mL), and 3.7 mg/mL of NaHCO<sub>3</sub>. Cells were cultured in a humidified atmosphere and incubated at 37°C in 5% CO<sub>2</sub>. Cultured cells were treated for 24, 48, and 72 h with 50 nM TCDD and various concentration of LRE (0, 50, 100, 200, and 400 µg/mL).

### Cell viability analysis

Cell counting kit-8 was used to determine cell viability, according to the manufacturer’s instructions. MCF-7 cells were seeded in a 96-well plate (1×10<sup>4</sup> cells/well) and incubated in Dulbecco’s modified Eagle’s medium at 37°C in 5% CO<sub>2</sub> for 24h. The cells were treated with TCDD and LRE (0, 50, 100, 200, and 400 µg/mL) for 24, 48, and 72h. After treatment, the medium containing LRE was removed and replaced with fresh medium containing 10 µL of CCK-8 solution and the plate was incubated at 37°C for 2h. Absorbance at 450 nm was measured with an ELISA plate reader. The viability of treated cells was expressed as percentage of that of control cells.

### Cell cycle analysis

Flow cytometry analysis was used to determine the proportion of MCF-7 cells at the different stages of the cell cycle. MCF-7 cells were seeded in 6-well plates at 2×10<sup>5</sup> cells/mL and incubated for 24h. The cells were then treated with TCDD and increasing concentrations of LRE (0-400 µg/mL) for 48 h. After treatment, the cells were harvested and washed twice with phosphate-buffered saline. Each sample was fixed in 1 mL of 70% ethanol for 2h at -20°C. After fixing, the samples were centrifuged, ethanol was removed, and the cells were resuspended in phosphate-buffered saline containing 50 µg/mL propidium iodide and 100 µg/mL RNase A and incubated in the dark for 30 min at room temperature. Cell cycle distribution was analyzed using the BD FACSCalibur flow cytometer. The obtained data were analyzed using the BD CellQuest Pro software.

### RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from TCDD- and LRE-treated cells using Trizol reagent according to the manufacturer’s protocol. RNA samples were reverse-transcribed with M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) and specific primers were used to amplify AhR, ARNT, cytochrome P450 1A1 (CYP1A1), p53, p27, cyclin-dependent kinase 1 (CDK1), cyclin A, and cyclin B1. The optimum number of cycles for each gene was determined experimentally. The housekeeping gene β-actin was used to verify that equal amounts of RNA were added to the PCR reaction. The expression levels of all genes were normalized to that of β-actin.

### Western blot

The cells treated with TCDD and 0-400 µg/mL LRE for 24h were lysed using a protein extraction solution (iNtRON Biotechnology). Total protein concentration was determined by the Bio-Rad protein assay. Next, 25 µg of protein was diluted and heated at 95°C for 10 min prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% and 12%). The proteins were

then transferred to nitrocellulose membranes and blocked overnight with 5% skim milk in TBST (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). The membranes were then rinsed four times with TBST and incubated for 2 h with 2% skim milk containing the primary antibody (diluted at 1:1000): p53, p27, CDK1, cyclin A, cyclin B1, AhR, ARNT, and CYP1A1. After washing four times with TBST buffer, the membranes were incubated for 2 h with the horseradish peroxidase-conjugated secondary antibody (diluted at 1:2000). The membranes were washed again and developed using enhanced chemiluminescence (ECL Western Blot Analysis System Kit, Amersham Biosciences).

### Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean±standard error. Differences between means were evaluated using one-way ANOVA followed by Duncan's multiple range test;  $p < 0.05$  was considered statistically significant.

## Results

### LRE inhibited TCDD-stimulated MCF-7 cell proliferation

First, we determined the effect of 50 nM TCDD on the viability of MCF-7 cells. As shown in Figure 1, TCDD stimulated the proliferation of MCF-7 cells. In contrast, when cells were treated with LRE, the survival curve showed that the cytotoxic effects of LRE on TCDD-treated cells were dose- and time-dependent. There was a significant decrease in cell viability of cells incubated for 24, 48, and 72 h. The data suggested that LRE significantly inhibited TCDD-induced MCF-7 cell proliferation in dose and time-dependent manners.

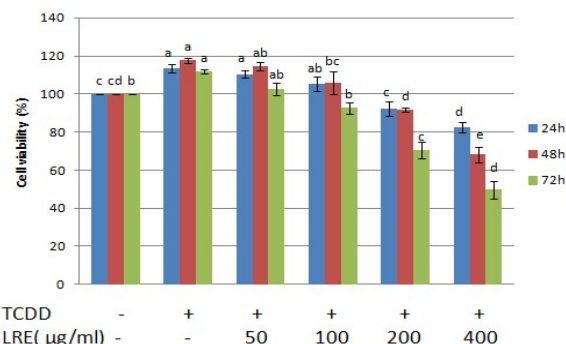
### LRE induced G2/M phase arrest in TCDD-treated MCF-7 cells

Flow cytometry analysis was performed to investigate whether LRE affected cell cycle regulation in TCDD-treated MCF-7 cells (Figure 2). DNA histogram analysis revealed that TCDD increased the percentage of cells in the G1/G0 phase. LRE treatment caused a dose-dependent

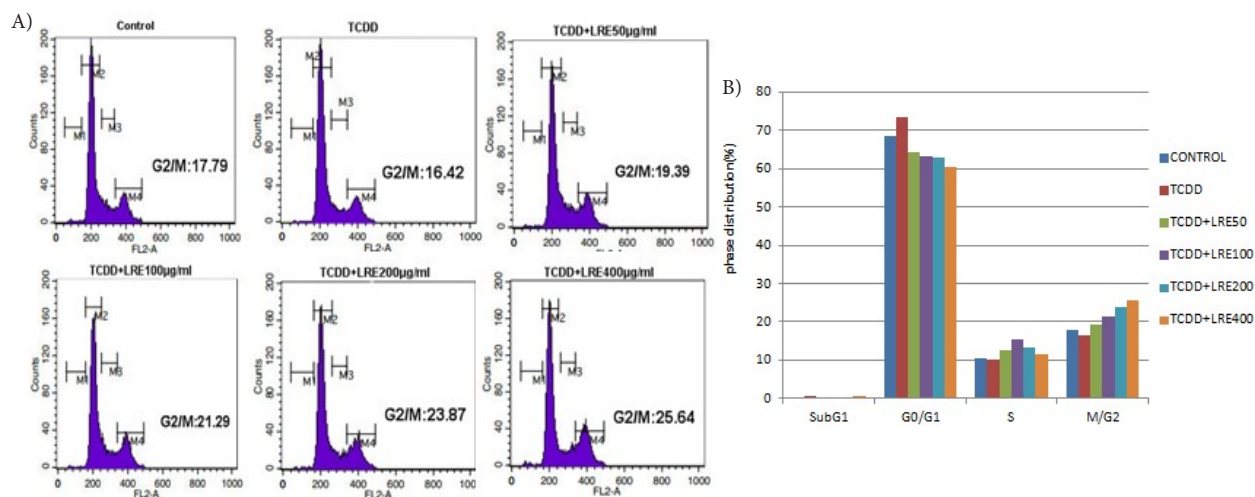
increase in the number of cells in the G2/M phase from 16.42% in the group treated with TCDD alone to 25.64% in the group treated with TCDD and 400  $\mu\text{g}/\text{mL}$  LRE. However, concomitant to this increase, a dose-dependent decrease in the percentages of cells in the G1/G0 phase (from 73.28% to 60.52%) and S phase (from 10.07% to 11.68%) was also observed in MCF-7 cells treated with TCDD and LRE, indicating that cell cycle progression from the G2/M phase was inhibited by LRE treatment. The data presented in Figure 2 clearly showed that LRE dose-dependently inhibited the proliferation of TCDD-treated MCF-7 cells via cell cycle arrest in the G2/M phase.

### Gene expression changes induced by LRE

To further understand the mechanism of LRE-induced cell cycle arrest in TCDD-stimulated MCF-7 cells, the expression levels of cell cycle-related genes were determined with reverse transcription-polymerase chain reaction (Figure 3A). Compared to TCDD-treated cells, the expression levels of p53 and p27 increased dose-dependently after LRE treatment for 24h. The expression levels of the cell cycle regulators CDK1, cyclin A, and cyclin B1 were decreased. Because TCDD, as an EDC, executes multiple biological activities primarily through AhR, we analyzed the gene expression of AhR, as well

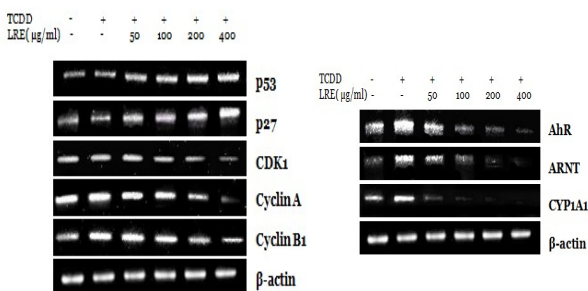


**Figure 1. Effects of Ethanol Extract of Licorice Root on the Proliferation of MCF-7 Cells Stimulated with 50nM TCDD.** 0.1% DMSO was used as a vehicle control. Data are means±SE (n=3). Means with different superscript are significantly different at  $p < 0.05$

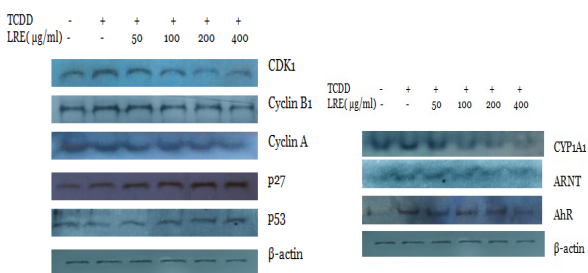


**Figure 2. The Effect of LRE on TCDD Treated MCF-7 Cell Cycle.** 0.1% DMSO was used as a vehicle control. (A) Cells treated with TCDD and different concentrations of LRE for 48hrs and analyzed by flow cytometry after staining with PI; (B) Histogram showing the number of cells in each phase of the cell cycle





**Figure 3. Effect of LRE on the mRNA Expressions of TCDD Toxicity and Cell Cycle Related Genes in TCDD Treated MCF-7 Cells.** 0.1% DMSO was used as a vehicle control



**Figure 4. Expressions of Cell Cycle Related Proteins in TCDD Stimulated MCF-7 Cells Treated with Increasing Concentrations of LRE.** 0.1% DMSO was used as a vehicle control

as ARNT and CYP1A1. The expression levels of these three genes were up-regulated by TCDD, whereas they were significantly down-regulated by LRE (Figure 3B).

*Effects of LRE on the expression of cell cycle- and TCDD toxicity-related proteins*

To investigate the possible molecular mechanism by which LRE triggered cell cycle arrest and mitigation of TCDD toxicity in MCF-7 cells, the protein expression of several cell cycle- and TCDD toxicity-related proteins in MCF-7 cells treated with LRE and TCDD was evaluated by Western blot analysis. Western blot data showed up-regulation of the tumor-suppressor protein p53, which controls cell growth through cell cycle arrest (p27 activation) and TCDD toxicity-related proteins (AhR, ARNT, and CYP1A1 inhibition).

**Discussion**

This study investigated the antiproliferative activity of LRE in TCDD-stimulated MCF-7 human breast cancer cells. LRE mitigated the tumorigenic effects of TCDD in MCF-7 cells by suppressing AhR expression and cell cycle arrest.

TCDD is an endocrine disruptor and its effects on AhR signaling are well known and have been demonstrated *in vitro* and *in vivo* (Jablonska et al., 2011; Hrabia et al., 2013). AhR is a ligand-activated transcription factor. It mediates most of the toxic responses of TCDD, including tumorigenic, immune, developmental, and endocrine effects (Mimura and Fujii-Kuriyama, 2002). Several studies in MCF-7 cells revealed that TCDD stimulates cell

proliferation through acceleration of cell cycle progression (Chen et al., 2012), which is in agreement with our results (Figure 1 and Figure 2). Licorice root is a widely used traditional Chinese herb that has already been identified by the National Cancer Institute (Craig, 1999) as having chemopreventive attributes. In our study, the cell viability assay showed that LRE exerted a potent cytotoxic effect on TCDD-stimulated MCF-7 cells. The inhibition of proliferation in TCDD-treated cells was a result of the suppression of AhR expression and cell cycle arrest, as evidenced by the down-regulation of the AhR signaling pathway that mediates the tumorigenic effect induced by TCDD; furthermore, FACS analysis of LRE-treated TCDD-stimulated MCF-7 cells showed cell cycle arrest in the G2/M phase (Figure 2).

Although the mechanism underlying TCDD-induced tumorigenesis is not completely understood, AhR seems to be a key transcriptional regulatory protein in TCDD-elicited gene expression. After specific binding of TCDD to AhR, the AhR-ligands translocate to the nucleus and heterodimerize with ARNT. Subsequently, the complex binds to the xenobiotic-response element, leading to the expression of dioxin-responsive genes, including CYP1A1 (Mimura and Fujii-Kuriyama, 2002). CYP1A1 is proved to have a carcinogen-activating role in terms of substrate specificity, mechanism of carcinogen activation, polymorphisms, and extrahepatic expression (Androutsopoulos et al., 2009; Chen et al., 2011). In the present study, we found that LRE suppressed the expression of AhR, ARNT, as well as CYP1A1 (Figure 3 and Figure 4), which are components of the primary signaling pathway that mediates the tumorigenic effect induced by TCDD.

The p53 protein is a tumor suppressor mainly involved in the transcriptional regulation of many growth arrest- and apoptosis-related genes (Levine, 1997). P53 plays an important role in stress response pathways that prevent proliferation and survival of potentially malignant cells (Ryan et al., 2001). P53 is crucial in multicellular organisms, and it has been described as “the guardian of the genome” because of its role in conserving stability by preventing genome mutation (Read et al., 1999). The activation of p53 contributes to cell-cycle arrest at the G1/S and/or G2/M checkpoints through diverse mechanisms (Wahl and Carr, 2001). Our results showed that LRE activated p53 and inhibited the growth and proliferation of MCF-7 cells treated with TCDD by cell cycle arrest.

P27 is a member of the CDK inhibitor family and encodes a putative tumor suppressor (Polyak et al., 1994). Many human tumors exhibit a variable loss of p27 protein and such loss has been described as an independent prognostic factor in various human cancers, including breast, colon, and prostate adenocarcinomas (Giordano et al., 2009). Indeed, p27 plays a direct role in mediating p53-induced G2/M phase arrest and p53 is its transcription factor (Casalini et al., 2007). P27, as a member of the CDK inhibitor family, regulates cell cycle progression through decreasing the expression of CDK1, cyclin B1, and cyclin A (Hu and Moscinski, 2010). In agreement with these studies, we observed that LRE up-regulated the expression

of p27 (Figure 3), down-regulated the activity of CDK1, cyclin B1, and cyclin A (Figure 3 and Figure 4), and then triggered cell cycle arrest in the G2/M phase.

In conclusion, the present study demonstrated that LRE possesses cytostatic properties by inducing cell cycle arrest and suppressing the expression of AhR in TCDD-stimulated MCF-7 cells. The results indicated that LRE could be used as a potential toxicity-alleviating agent to prevent the occurrence of EDC-induced tumors.

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