Original Article

Effect of Genistein and 17-β Estradiol on the Viability and Apoptosis of Human Hepatocellular Carcinoma HepG2 cell line

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Abstract

Background: One of the most lethal cancers is hepatocellular carcinoma (HCC). Genistein (GE) is a choice compound for treatment of certain types of cancer. Phytoestrogens are plant derivatives that bear a structural similarity to 17-β estradiol (E2) and act in a similar manner. They are a group of lipophillic plant compounds with tumorigenic and antitumorigenic effects. E2 has stimulatory and inhibitory effects on cancer cell lines. This study was designed to investigate the antiproliferative and apoptotic effects of GE and E2 on the HCC HepG2 cell line. **Materials and Methods:** HepG2 cells were cultured and treated with various concentrations of GE and E2 and then 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromideand flow cytometry assay were performed to determine cell viability and apoptosis. **Results:** GE and E2 induced apoptosis and inhibited cell growth significantly. Reduction of cell viability by 50% required 20 μM E2 for E2-treatment groups and 20 μMGE for GE-treatment groups. The percentage of the GE-treated apoptotic cells was reduced by about 35%, 42%, and 47% ($P < 0.001$) and that of E2-treated groups 34%, 39%, and 42% ($P < 0.001$) after 24, 48, and 72 h, respectively. **Conclusions:** Our experimental work clearly demonstrated that GE and E2 exhibited significant antiproliferative and apoptotic effects on human HCC HepG2 cells.

Keywords: *17-β estradiol, apoptosis, genistein, hepatocellular carcinoma, proliferation*

Introduction

One of the most lethal cancers is hepatocellular carcinoma (HCC).[1] The cancer is a slow process during which genetic and epigenetic changes progressively alter the genes expression evolving in prevention of HCC and is chemoresistant to most currently available chemotherapeutic agents. Liver cancer is one of the most common causes of cancer deaths worldwide and the incidence of this fatal disease is correlated with the presence of infection with hepatitis viruses.[2] Genistein (GE), an isoflavinoid in soy beans, is a choice compound for treatment of certain types of cancer such as gastric cancer, prostate cancer, breast cancer, and colon cancer.[3] In the previous studies, we reported that GE can induce apoptosis in PLC/PRF5 and HepG2 HCC cell lines.[4,5] Phytoestrogens, a group of natural compound, have estrogen-like activity and similar structure to estradiol (E2) originating from various plant sources include fruits, soy beans, vegetables, legumes, and flax seeds. These compounds are phenolic nonsteroidal plant-derived compounds possessing estrogen-like activity.[6] Phytoestrogens are plant derivatives that bear a structural similarity to E2 and act in a similar manner. They are a group of lipophillic plant compounds with tumorigenic and anti-tumorigenic effects[7] that demonstrate weak estrogenic and antiestrogenic activity in different tissues. Really, their tumorigenic and antitumorigenic effects depend on their concentrations.[8]

Many studies have demonstrated that E2 has stimulatory effect on the proliferation of human WRO, FRO, and ARO thyroid carcinoma cells.[9] Other results have shown that E2 significantly increases apoptosis in prostate, MDA-MB-231 breast, colorectal and pancreatic cancers.[10,11] Our previous work indicated that E2 can inhibit proliferation and induce apoptosis in PLC/PRF5 HCC cells.[12] However, only limited studies are available to report the effects of GE associated with E2 on HCC HepG2 cells. This study was designed to investigate the antiproliferative and

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apoptotic effect of GE and E2 on the HCC HepG2 cell line.

Materials and Methods

Materials

HepG2 cells were purchased from the National Cell Bank of Iran-Pasteur Institute. Dulbecco minimal essential medium (DMEM), 17-β E2, GE, AnnexinV-FITC, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (Sigma, St. Louis, MO, USA). All other compounds including fetal bovine serum (FBS) (product number f2442), penicillin (CAS number 69-57-8), and streptomycin (CAS number 3810-74-0) obtained from sigma too. 17-β estradiol and GE were dissolved in dimethyl sulfoxide (DMSO); DMSO was present at 0.01%–0.3%.

Cell culture and treatment

As mentioned above, HepG2 cells were purchased from the National Cell Bank of Iran-Pasteur Institute and cultured in Dulbecco's Modified Eagle Medium (DMEM). All experimental media were supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The HepG2 cells were incubated at 37°C, 95% humidity, 5% CO_2. When HepG2 cells became >80% confluent, 5×10^5 cells were cultured into 24-well plates (Becton-Dickinson) for 24 h in culture medium before they were incubated with certain concentrations of E2 (1, 5, 10, 25, 50, 75, and 100 μM) and GE (1, 5, 10, 25, 50, 75, and 100 μM) dissolved in DMSO. It should be noted that the control groups were treated with DMSO only. The proliferative effects of certain concentrations of GE and E2 (as mentioned) were assessed by MTT assay, according to the standard protocols. After 24, 48, and 72 h of treatment, the HepG2 cells were washed twice with phosphate-buffered saline, and then a fresh medium containing MTT (0.5 mg/mL) was added. After 4 h, the formazan crystals were dissolved in acidic isopropanol and the absorbance was measured at 540 nm. All experiments of the all groups were repeated three times, with at least three measurements (triplicates).

Determination of apoptotic cells by flow cytometry assay

Human HCC HepG2 cells were seeded in 24-well plates. After 24 h of culture time, the medium-free chemical was changed with medium contains 20 μM E2 (in the three groups) and 20 μM GE (in the other three groups), obtained average dose as means of 24, 48, and 72 h. Flow cytometry assay was down after 24, 48, and 72 h of treatment. Besides, two groups were received combined drugs; first treated with GE (20 μM) and after 24 h treated with E2 (20 μM) and finally 24 and 48 h after treated with E2 flow cytometry assay was down. In this method, all the HepG2 adherent cells were trypsinized by 0.05% trypsin and then Annexin-V-(FITC) and propidium iodide (PI, Becton-Dickinson, San Diego, CA, USA) were used for staining of collected cells according to the manufacturer's instructions. The double-stained HepG2 cells were analyzed by a FACSCanto flow cytometer (Becton-Dickinson, Mountain View, CA, USA). All experiments of this study were processed independently three times. In each experiment, a minimum of 5×10^5 cell/ml were analyzed.

Statistical analysis

The database was setup with the SPSS 16.0 software package for analysis. The data were acquired from three tests and are shown as means ± standard deviations. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA) and Tukey's test. A significant difference was considered $P < 0.05$.

Results

Result of the MTT assay

The proliferative effects of GE and E2 with different concentrations (as mentioned above) in 24, 48, and 72 h were analyzed using the MTT assay. The amounts of reduced MTT in the all experimental groups were significantly lower than that of the control groups ($P < 0.001$). E2 inhibited growth in all treated groups by 88%–32% at 24 h, 82%–30% at 48 h, and 72%–22% at 72 h ($P < 0.001$) and also GE inhibited growth in all treated groups by 80%–30% at 24 h, 72%–24% at 48 h, and 64%–18% at 72 h ($P < 0.001$). Reduction of cell viability by 50% (IC50) required 20 μM E2 for E2-treatment groups and 20 μM GE for GE-treatment groups. These IC50 doses of GE and E2 are average doses of different time periods (24, 48, and 72 h). For consistency of the result, each experiment was repeated three times [Figure 1].

Result of the apoptosis assay

The HepG2 cells were treated with alone and combined drugs, 20 μM E2 and 20 μM GE (obtained average dose as means of 24, 48, and 72 h), for different time periods (24, 48, and 72 h) and flow cytometry was performed to determine the apoptotic cells. As shown in Figure 2, flow cytometry revealed that GE and E2 (alone and combined) induced significant apoptosis versus control group. Maximal apoptosis induction was observed in the group which received GE (24 h) and then E2 (48 h) and minimal apoptosis induction was observed in the group which received E2 alone for 24 h.

The percentage of GE-treated apoptotic cells were reduced by about 35, 42, and 47% ($P < 0.001$) and that of E2-treated groups 34, 39, and 42% ($P < 0.001$) after 24, 48, and 72 h, respectively. In the flow cytometry graph, the right lower quadrant was considered as primary apoptotic cells and the right upper quadrant as secondary apoptotic cells. Maximal apoptotic cell (62%, $P < 0.001$) was observed in the group which received GE for 24 h and then E2 for 48 h and minimal apoptotic cell (35%, $P < 0.001$)

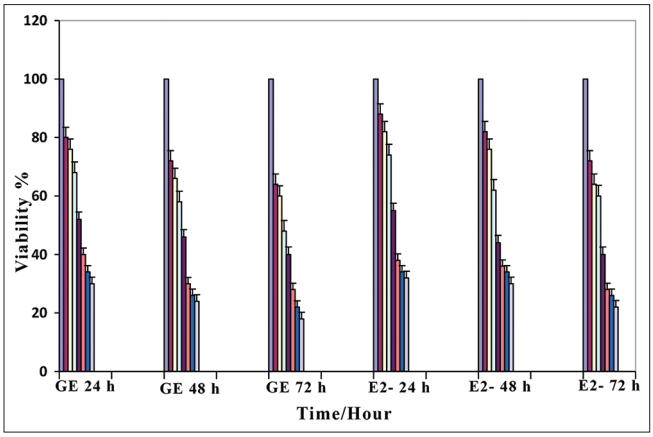

Figure 1: The cell vitality in the cells which treated with genistein and estradiol at mentioned concentration in different time periods (24, 48 and 72 h) analyzed by using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. In each group from left to right, first column belongs to control and others belong to 1, 5, 10, 25, 50, 75 and 100 μM concentrations respectively. The amounts of reduced 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide in the all experimental groups were significantly lower than that of the control groups (*P* < 0.001)

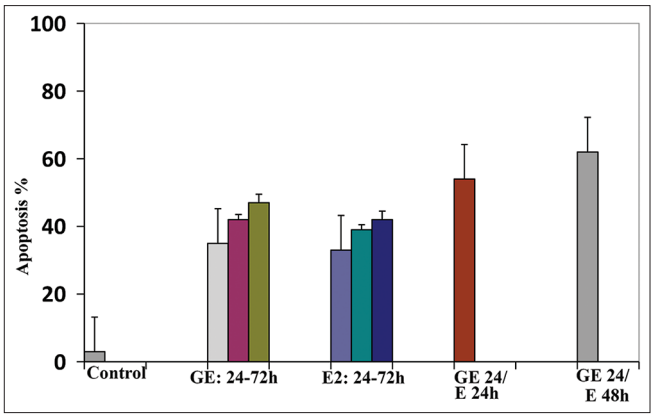

Figure 2: Effect of genistein and estradiol on HepG2 cells apoptosis. The cells were treated with genistein (20 μM) and estradiol (20 μM) for 24, 48 and 72 h and the apoptosis- inducing effect were investigated by flow cytometric analysis of HepG2 cells stained with Annexin V and propidium iodide. Genistein and estradiol (alone and combined) induced significant apoptosis in the all treated groups versus control group (*P* < 0.001), 95% confidence interval

was observed in the group which received E2 alone for 24 h [Figures 2 and 3].

Discussion

HCC is a global health problem and one of the most causes of cancer-related death. Chronic hepatitis B and C are well-recognized risk factors of HCC. GE, a flavonoid and a bioactive component of soy isoflavones, is a potent apoptosis inducer of human cancers. Experimental and epidemiologic studies have reported that soyfoods prevent cancer and induce apoptosis in many different organs. Many researchers have indicated that the isoflavonoid GE is one of the most components responsible for apoptosis in different cancers.[13] Phytoestogens, one group of plant compounds, have estrogenic effects in animals; both tumorigenic and antitumorigenic effects have been reported. Regulation of the growth and differentiation of many tissues are one of the actions of the estrogens and these compounds can act as mitogen.[14] It have been demonstrated that material nutrition rich in phytoestrogen can influence cancer development and cancer inhibition.[15]

Our experimental work showed that E2 and GE (alone and combined) can inhibit proliferation of HepG2 cell and induce apoptosis in this cell line. Significant inhibitory and apoptotic effects of GE on the HepG2 cell have been reported in the other works[14] and also same effects of E2 on the PLC/PRF5 cell line.[12] Therefore, in this work, we evaluated the effects of E2 and GE (alone and combined) on HepG2 cell line. We expected agonistic effect of E2 in this work, but it showed antagonistic effect in this cell line. Similar study has been reported that GE has apoptotic and antiproliferative effect in MDA-MB-435 and MDAMB-231 breast cancer cell line.[16] Antimetastatic effect of GE on the prostate cancer cell has been reported by other researchers too.[17] Similarly, apoptotic effect of E2 on prostate and colorectal cancer cell has been shown.[11]

In vivo study has been shown that E2 induces apoptosis in prostatic cancer in the rat.[18] GE can induce apoptosis by different mechanisms. One of the mechanisms by which GE acts is upregulation of p21 and downregulation of PLK-1 reported in LNCaP and PC-3 cells.[19] There are various experimental evidences showing that GE is a protein tyrosine kinase inhibitor[20] and inhibits cancer cell growth by modulation of the genes, which involve in apoptosis. It has been reported that GE inhibits Nuclear factor-kappa B (NF-κB) and Akt signaling pathways which play an important role in the cell viability.[21] It can inhibit transcription of the NF-κB-dependent genes by preventing NF-κB from binding to its target DNA.[22] It has demonstrated that GE induces apoptosis by Bcl-2 and Bax induction in estrogen receptor (ER)-positive MCF-7 cells.[23] Various pathways have been reported for apoptotic effect of E2. Experimental works have been indicated that E2 potentiates prostaglandin J2 (PGJ2)-induced apoptosis in breast cancer MCF-7 cell and has an additive effect on PGJ2-induced cell apoptosis.[24] E2 induces apoptosis through intrinsic pathway of mitochondrial disruption and release of cytochrome C.[25] Activation of the Fas/FasL pathways has been reported as a mechanism by which E2 induces apoptosis in breast cancer cells.[26] Similarly, a link between estradiol-induced apoptosis and activation of the FasR/FasL death-signaling pathway have been reported.[27] Other researchers have indicated that E2 bind to cell surface proteins resulting in calcium reflux and activation of adenylate cyclase and phospholipase C and finally cAMP and IP3 generation and also demonstrated that stimulation/inhibition (dependent on the ER subtype) of phosphoinositol-3 hydroxy kinase and the family of mitogen-activated protein kinases, such as p38β isoform and c-Jun N-terminal kinase (JNK) are rapidly responsive

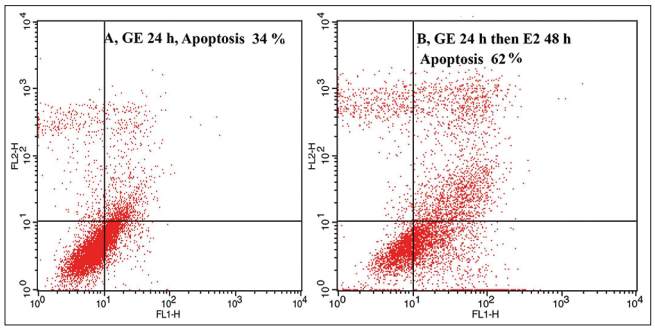

Figure 3: Effect of genistein on HepG2 cells apoptosis. Maximal apoptotic cell was observed in the group which received genistein for 24 h and then estradiol for 48 h and minimal apoptotic cell was observed in the group which received E2 alone for 24 h

to E2 that regulate migration, angiogenesis, and apoptosis through nuclear compartments.[28-31]

The above-mentioned reports support our result. Opposite to our report, it has been shown that E2 stimulates proliferation of prostate stromal cells (PrSCs).[32]

Proliferative effect of E2 on breast cancer MCF-7 cells, human colon carcinoma HT-29 and Caco-2 cells lines have been reported too.[33]

On the contrary to our report, several studies have reported that E2 has proliferative effect, E2 induces proliferation in gastric cancer cell[34] and also accelerates ER-negative breast cancer metastasis and increases metastatic tumor and colony formation in the lungs in mice.[35] Similar work has indicated that E_2 stimulates proliferation of primary prostate stromal cells (PrSCs and WPMY-1)[32] and human breast epithelial cell MCF-10F.[36] In the present study, we reported apoptotic effect for GE but just the opposite several studies have demonstrated proliferative effect for this compound. It has been reported that GE stimulates the proliferation of MCF-7 and T47D breast cancer cells[37] and also MCF7wt and MCF7SH breast cancer cells.[38] This compound has proliferative effect on mammary gland too.[39] *In vivo* study has demonstrated that GE accelerates prostate cancer progression in TRAMP-FVB mice.[40] Finally, these compounds have biphasic effects and can inhibit proliferation or induce apoptosis according to dose, time, and tissue. In this work, we did not related experiments about acetylation and methylation involved in apoptosis.

Conclusions

Our result suggests that E2 and GE (alone and combined) may be a potent anticarcinogenic compounds and can effectively inhibit proliferation and induce apoptosis in HepG2 cells.

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Conflicts of interest

There are no conflicts of interest.

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