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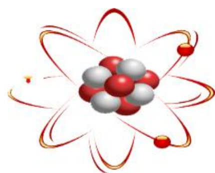

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GENISTEIN SENSITIZES EFFECT OF TAMOXIFEN ON HUMAN HEPATOCELLULAR CARCINOMA CELL LINE

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ABSTRACT

Genistein (GE) has been shown to suppress the growth of various cancers through modulation of various pathways , in particular, reactivation of estrogen receptor alpha (ERα). This compound significantly decreases methylation of DNA promoter by reduction of DNMT1 activity . Tamoxifen(TAM) alters steroid binding domain which prevents gene activation and affects the tumor growth . GE and TAM have significant anti-tumor effects .The aim of the present study was to analyse the effects of GE on ERα and DNMT1 expression and also apoptotic and antiprolifrative effects of GE and TAM on hepatocellular carcinoma . The cells were incubated with certain concentrations of GE (1, 5, 10, 25, 50, 75, and 100 µM) and the same concentrations of TAM and MTT assay were performed to assess cells viability. Apoptotic effects of these compounds with 25 µM concentration (alone and combined) were measured by using flow cytometry at different times (24, 48 and 72 h).The expression level of ERα and DNMT1 were determined by quantitative real-time RT PCR. our result demonstrated that , GE increases ERα and decreases DNMT1 gene expression , GE and TAM inhibit cell viability and induce apoptosis significantly . The cell viability was decreased and the apoptotic cells were increased significantly but combined compound induced apoptosis more significantly. The relative expression of ERα in GE (25 µM) treatment group were increased and that of DNMT1 were decreased significantly. According to our results, GE can epigenetically increase ERα expression by inhibition of DNMT1 expression which in turn increases apoptotic effect of tamoxifen. Furthermore, combination of GE and TAM induce apoptosis more significantly and also GE and TAM inhibit proliferation significantly in a dose- and time-dependent manner.

Keywords: GE, TAM , ERα, DNMT1, Epigenetic, Hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma , is one of the most common type of malignancy and the major form of primary liver cancer. It is the sixth most prevalent cancer in the world and the third leading cause of cancer related mortality [1]. This disease is a main complication of cirrhosis and typically starts with a pre-existing liver disease caused by infection with hepatitis C virus (HCV) , hepatitis B virus (HBV) or alcohol consumption [2]. It is associated with a poor prognosis due to a delay in diagnosis and complex pathogenesis because of involving different molecular pathways. Carcinoma arises from both, epigenetic and genetic alterations [3] that change gene expression and cell function [4]. Epigenetic alterations are reversible by epi-drugs which are drugs targeting epigenetic mechanisms can reverse epigenetic alternation[5,6,7]. DNA methylation, the covalent addition of a methyl group to the 5´ position of cytosine, is the most important known form of epigenetic information in mammalian cells [8,9,10] and DNA hypermethylation plays an important role in silencing the tumor suppressor genes that lead to an abnormal activation or inactivation of multiple cellular signaling pathways including cellular proliferation, cellular survival, cellular differentiation, and angiogenesis. Aberrant epigenetic changes are consistently associated with different cancer types, including lung cancer, colorectal cancer and HCC [2] .

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DNA methyltransferases (DNMTs), a family of enzymes that is encoded by DNMT genes in the human genume, catalyze DNA methylation . There are four types of DNMT genes contain DNMT1, DNMT2, DNMT3A, and DNMT3B (11) that DNMT1 is the most abundant DNA methyltransferase and considered to be the key maintenance methyltransferase in the mammals.

Tumor suppressor genes and epigenetic pathways have opened a new window on cancers treatment and after identifying the receptors which present on cancer cells surface , the role of these receptors has been confirmed in various cancers , hence, the function of these receptors is target of treatment programs.

Estrogen receptor gene is one of the genes that has recently attracted the attention of researchers and its role in various cancers has been demonstrated. This gene has two types include ESR1 and ESR2 genes that located on chromosomes 6q25.1 and 14q22-24 and code ERα and ERβ respectively [12].

These receptors are very important choices for endocrine therapy. Both types of receptors are expressed in many tissues including the nervous system, cardiovascular, breast and bone tissues and also ERα is expressed in liver tissue [13].

GE (4',5,7-trihydroxyisoflavone), a major isoflavone constituent of soybeans and soy products, has been shown to suppress the growth of various cancers such as ovarian , oesophagus , breast , lung and colon cancers through modulation of various pathways , in particular, reactivation of ERα . GE has multiple molecular targets including receptors, enzymes, and signaling pathways but often acts via ER [14]. This compound significantly decreases methylation of DNA promoter by reduction of DNMT1 activity . Many studies have indicated that the incidence and mortality rates of these cancers are considerably lower in Asia compared to the United States because of consumption of soybeans and soy products [15]. It has been demonstrated that the inhibition of DNA methyltransferase activity can strongly inhibit the formation of cancers [14]. Tamoxifen (TAM) has been used for treating breast cancer For many years . This compound changes steroid binding domain which prevents gene activation and affects tumor growth. GE and TAM have significant anti-tumor effects [16], therefore, combination of them may be a good candidate for the treatment of HCC.

In this study, we investigated whether GE could alter the ERα and DNMT1 expression and also investigated apoptotic and prolifrative effects of GE and TAM on PLC/PRF5 hepatocellular carcinoma cell line .

MATERIALS AND METHODS

Materials

Human hepatocellular carcinoma cell line (PLC/PRF5) was purchased from the National Cell Bank of Iran-Pasteur Institute. GE, TAM, Total RNA extraction Kit (TRIZOL reagent), Real-time PCR kits (qPCRMasterMix Plus for SYBR Green I dNTP), DMEM (Dulbecco's modified Eagle's medium Nutrient Mixture F-12 Ham) and MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma (Sigma, St. Louis, MO). All other chemicals were obtained from the best available sources.

Cell culture

The cells were cultured and grown in DMEM supplemented with 10% fetal bovine serum. The cultures were incubated at 37°c in a humidified incubator containing 5% CO_2, 95% ambient air. When cells became >80% confluent, 5×10^5 cells (PLC/PRF5) were seeded into 24-well plates (Becton-Dickinson) for 24 h in DMEM culture medium before they were incubated with certain concentrations of GE (1, 5, 10, 25, 50, 75, and 100 μM/lit) , which was dissolved in dimethyl sulfoxide (DMSO); DMSO was present at 0.01–0.3% in the medium. After 24 h, culture medium was changed with culture medium contains various concentrations of GE. On days 2, 3 and 4 after treatment with GE, MTT assay was done . The MTT assay for determination of IC50 value for TAM was done as done for GE with certain concentrations of TAM (1, 5, 10, 25, 50, 75, and 100 μM/lit) which was dissolved in dimethyl sulfoxide (DMSO); DMSO was present at 0.01–0.3% in the medium . Photography was done for cultures before and after treatment with GE and TAM at different times using inverted microscope (Nikon, TE 2000-U, Japan).

Determination of IC50 Value by MTT Assay

After 24, 48 and 72 h of the treatment, the IC50 value for GE and TAM in PLC/PRF5 group were determined. Briefly, 5×10^5 Cells (PLC/PRF5) were counted and placed into each well of a 24-well culture plates. After 24h of seeding, various concentrations of GE and TAM were added to the cells except in the control groups and after 24, 48 and 72 h of drug exposure, the MTT survival assay was then carried out for the evaluation of the cell viability with different drug concentration. The cells measured spectrophotometrically at 570 nm .All experiments were repeated three times, with at least three measurements (triplicates).

Determination of Cell Viability By MTT Assay

The MTT assay was commonly used to assess cell proliferation and viability by measuring the reduction of yellow MTT by mitochondrial dehydrogenases in viable cells. This yields purple formazan crystals that detected colorimetrically at 570 nm.

To determine the effect of GE and TAM, the cells were seeded in triplicate in 24-well plates and treated with GE and TAM at concentration of 25 μM in different

period times (24, 48 and 72 h). The absorbance of the cell lysates in DMSO solution was read at 570 nm by a microplate reader (Bio-Rad Hercules, CA).

Determination of Apoptotic Cells By Flow Cytometry Assay

The cells were cultured in 24-well culture plates and divided into eleven groups after 24 h. Three groups received single dose of GE at the concentration of 25μM and also three groups received single dose of TAM at the concentration of 25μM for 24 , 48 and 72h respectively . One group received GE (25 μM) for 24 h and followed by TAM (25 μM) for 24 h (total treatment time 48 h) and other group received same dose of GE (GE) for 48 h and followed by TAM (25 μM) for 24 h (total treatment time 72 h). Final three groups received DMSO as control groups. In the GE treated groups (three groups) , TAM treated groups (three groups) after 24 , 48 and 72 h and GE-TAM groups (two groups) after 24h of TAM treatment and also control groups , all the adherent cells were collected with 0.05% trypsin, washed with cold phosphate-buffered saline (PBS) and re suspended in Binding buffer (1x). After addition of AnnexinV-FITC and propidium iodide (PI, Becton-Dickinson, San Diego, CA), analysis was carried out according to the manufacturer's protocol (BMS500F1/100CE AnnexinV-FITC, eBiscience, USA). Finally the apoptotic cells were counted by FACS can flow cytometry (Becton Dickinson, Heidelberg, Germany). All experiments were processed independently three times. A minimum of 5×10^5 cell/ml were analyzed for each sample.

Determination of Gene Expression By Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR amplification and analysis were achieved to quantitatively estimate the expression of ERα and DENMT1 in GE(25μM)-treated PLC/PRF5 cells at different times . Total RNA was isolated by RNeasy mini kit (Qiagen) according to the manufacturer's protocol and then treated by RNase free DNase (Qiagen) to eliminate the genomic DNA. The RNA concentration was determined using a Biophotometer (Eppendorf). Total RNA (100 ng) was reversetranscribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Real-time RT-PCR was performed by the Maxima SYBR Green RoxqPCR master mix kit (Fermentas). ERα and DNMT1 primers were obtained from articles [11,17,18] which their sequences are shown in Table1. Real-time PCR reactions were performed using the Steponeplus (Applied Biosystem). Thermal cycling conditions for ERα was : an initial denaturation at 95 ˚ C for 10 minutes, followed by 40 cycles of denaturation at 95˚ C for 20 seconds, annealing at 58 ˚c for 15 seconds and extension at 72 ˚ C for 15 seconds . Thermal cycling conditions for DNMT1 was: an initial denaturation at 95°C for 10 minute followed by 40 cycles of denaturation at 95˚C for 15 seconds, annealing at 60˚c for 20 seconds and extension at 72˚c for 20 seconds. Data were analyzed using the comparative Ct (ΔΔct) method, the relative expression level of ERα and DNMT1 were calculated by determining a ratio between the amount of these genes and that of endogenous control. Melting curve was used to determine melting temperature of specific amplification products and primer dimmers. These experiments were carried out in triplicate and independently repeated at least three times. GAPDH was used as a reference gene for internal control.

Result of Determination Of IC50 Value By MTT Assay

The effects of the GE and TAM on the cell viability after exposure with various concentrations (as mentioned) were assessed by MTT assay. Dose- and time-dipendent antiproliferative effects were observed with IC50s for TAM and GE. Reduction of cell viability by 50% (IC50) required 25 μm GE for GE-treatment groups and same dose for TAM-treatment groups at different times. Each experiment was repeated three times for consistency of the result. The Percentage of cell viability for GE(25μM)-treatment groups were 49% ($P < 0.002$) ,44 % ($P < 0.02$) and 43% ($P < 0.001$) and for TAM(25μM)-treatment groups were 50 % ($P < 0.001$),46 % ($P < 0.001$) and 44% ($P < 0.001$) at different time (24,48 and 72h) respectively (Fig.1,2) .

The cells were treated without and with different concentrations of GE for 24, 48 and 72 h. Each experiment was conducted in triplicate. Mean values from the three experiments ± standard error of mean (SEM) are shown. $^{*}P$, $^{**}P$, $^{***}P < 0.021$.

The cells were treated without and with different concentrations of TAM for 24, 48 and 72 h. Each experiment was conducted in triplicate. Mean values from the three experiments ± standard error of mean (SEM) are shown. $^{*}P$,$^{**}P$, $^{***}P < 0.001$.

Result of Determination of Cell Viability By MTT Assay

The cell vitality in the cells which treated with GE and TAM at concentration of 25 μM in different times were analysed by using the MTT assay. The amounts of reduced MTT in the all groups treated with GE and TAM were significantly lower than that of the control group ($P < 0.001$).The Percentage of cell viability for GE(25μM)-treatment groups were 49% ($P < 0.002$) ,44 % ($P < 0.02$) and 43% ($P < 0.001$)(fig.3) and for TAM(25μM)-treatment groups were 50 % ($P < 0.001$),46 % ($P < 0.001$) and 44% ($P < 0.001$)(fig.4) at different times (24,48 and 72h) respectively. There is a significant difference between percentage of cell viability of GE treatment groups in 24h and 72 h ($P < 0.001$) .In TAM treatment groups there are significant differences between percentage of cell viability of all experimental groups ($P < 0.04$).

The amounts of reduced MTT in the all groups treated with GE were significantly lower than that of the

control group (P < 0.001).The Percentage of cell viability for GE(25µM)-treatment groups were 49% (P < 0.002) ,44 % (P < 0.02) and 43% (P < 0.001) at different times (24,48 and 72h) respectively. There is a significant difference between percentage of cell viability of GE treatment groups in 24 h and 48 h (P < 0.04) but there isn't any significant difference between percentage of apoptosis of GE treatment groups in 48 h and 72 h (P < 0.25) .

The amounts of reduced MTT in the all groups treated with TAM were significantly lower than that of the control group (P < 0.001).The Percentage of cell viability for TAM(25µM)- treatment groups were 50 % (P < 0.001),46 % (P < 0.001) and 44% (P < 0.001) at different times (24,48 and 72h) respectively. There is a significant difference between percentage of cell viability between different experimental groups (P < 0.04).

Result of Determination of Apoptosis By Flow Cytometry Assay

The apoptosis-inducing effect of GE and TAM were investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidiumiodide. We observed via flow cytometry that these compounds induce apoptosis in this cell line significantly. The percentage of apoptotic cells in GE(25µM)-treatment group at different times (24,48 and 72h) were 49,55,56 % (P < 0.001) (fig . 5) and in TAM (25µM)-treatment group at different times (24,48 and 72h) were 38,40,46 % (P < 0.001) (fig 6) respectively. The percentage of apoptotic cells in the group that was treated with GE(25µM) for 24 h and followed by TAM (25 µM) for 24 h was 60 % and in the group that was treated with GE(25µ) for 48 h and followed by TAM (25 µM) for 24 h was 73 % (p<0.001) (fig. 7) . Relative analysis between GE treatment groups and TAM treatment groups at different times indicated that GE induces apoptosis more significantly (P< 0.001)(fig.8).

The percentage of apoptotic cells in the groups that treated with combined compound were significantly higher than that of the experimental groups that treated with GE or TAM alone , with 60 % and 73 % apoptotic cells respectively as shown in the fig.9 ($^{*}P < 0.003$, $^{**}P < 0.001$). Apoptotic effect were not observed in DMSO control group . A minimum of 5×10^{5} cells/ml were analyzed for each sample. Results was obtained from three independent experiments and were expressed as mean ± S.E.M.

Result of flow cytometry indicated that, GE induces cell apoptosis in PLC/PRF5 cells. The percentage of apoptotic cells in GE (25µM)-treatment groups at different times (24,48 and 72h) were 49, 55, 56 % (P < 0.001) respectively . Results were obtained from three independent experiments and were expressed as mean ± S.E.M. n=3.(A) 24 h. (B) 48 h . (C) 72 h.

Result of flow cytometry indicated that, TAM induces cell apoptosis in PLC/PRF5 cells. The percentage of apoptotic cells in TAM (25µM)-treatment groups at different times (24, 48 and 72h) were 38, 40, 46 % (P < 0.001) respectively. Results were obtained from three independent experiments and were expressed as mean ± S.E.M. n=3. (A) 24 h. (B) 48 h . (C) 72 h.

Combination of GE and TAM induced cell apoptosis in PLC/PRF5 cells significantly. The percentage of apoptotic cells in the group that was treated with GE(25µM) for 24 h and followed by TAM (25 µM) for 24 h was 60 % and in the group that was treated with GE(25µ) for 48 h and followed by TAM (25 µM) for 24 h was 73 % (P < 0.001). Results were obtained from three independent experiments and were expressed as mean ± S.E.M. n=3. (A) GE 24 h/TAM 24 h. (B) GE 48 h / TAM 24 h . 48 h. (C) control.

Result of Determination of Gene Expression By Real-Time Quantitative RT-PCR

To characterize the effect of genistein on PLC/PRF5 mRNA expression, time-course experiments were performed (24, 48 and 72 h). Using quantitative RT-PCR, GE was shown to significantly increase ER expression (fig.10) and decrease DNMT1 expression (fig.11) at different times. The relative expression of ERα were 1.8, 2.6 and 2.8 (P < 0.006) and expression of DNMT1 were 0.27 ,0.22 and 0.18 (P < 0.001) in different times respectively . In conclusion, GE increases ERα expression and decrease DNMT1 expression significantly as shown in fig.12.

Quantitative RT-PCR analysis demonstrated that, GE increased ERα expression significantly . Data are presented as means ± S.E.M. P < 0.006. n=3

Quantitative RT-PCR analysis demonstrated that, GE decreased DNMT1 expression significantly . Data are presented as means ± S.E.M. P <0.001, n=3.

Data are presented as means ± S.E.M. P < 0.001, n=3.

DISCUSSION

Hepatocellular carcinoma is the most common malignancy of liver diseases that carcinogenic agents and viral infections are known as its risk factors [2]. The disease is more common in men than women [19] and the level of ERα gene expression is involved in the disease [13]. It is known that, epigenetic regulation of genes play an essential role in the etiology of cancers. DNA methylation is an important epigenetic event in the regulation of gene expression and cell function.

Table 1. Real time polymerase chain reaction primers used in the study

Genes	Primer sequences
ERα	
Forward :	5′-AGA CAT GAG AGC TGC CAA CC-3′
Reverse :	5′-GCC AGG CAC ATT CTA GAA GG-3′
DNMT1	
Forward :	5′-TAC CTG GAC GAC CCT GAC CTC-3′
Reverse :	5′-CGT TGG CAT CAA AGA TGG ACA-3′

Figure 1. Effect of GE on viability of hepatocellular carcinoma cell line determined by MTT assay3

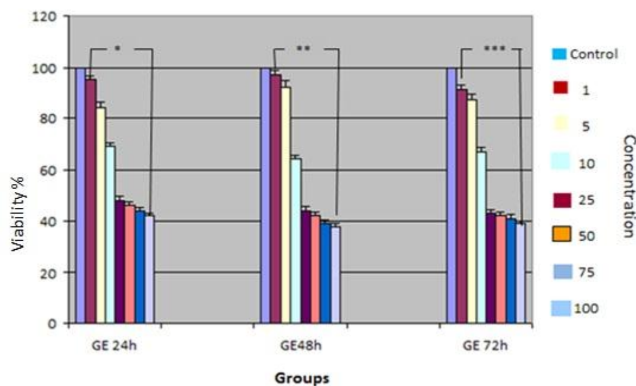


Figure 2.Effect of TAM on viability of hepatocellular carcinoma cell line determined by MTT assay

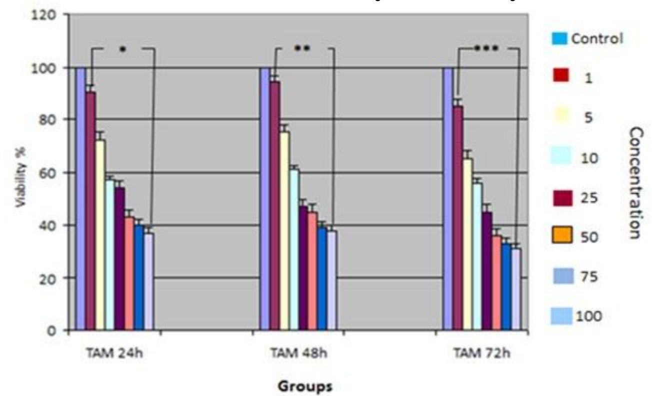


Figure 3. The cell vitality in the cells which treated with GE at concentration of 25 µM in different times were analysed by using the MTT assay

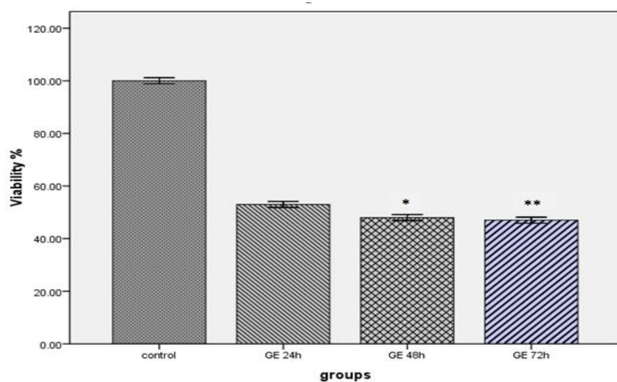


Figure 4. The cell vitality in the cells which treated with TAM at concentration of 25 µM in different times were analysed by using the MTT assay

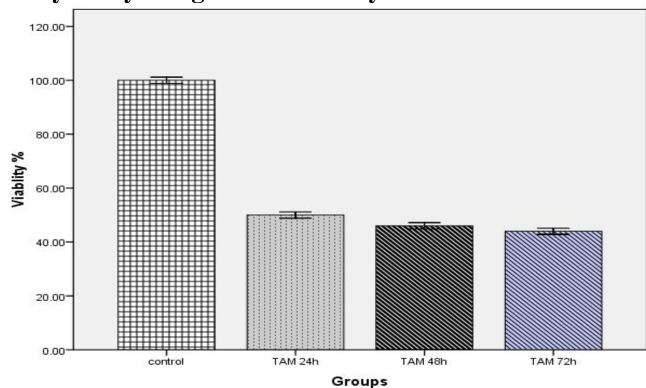


Figure 5. The apoptosis-inducing effect of GE was investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidium iodide

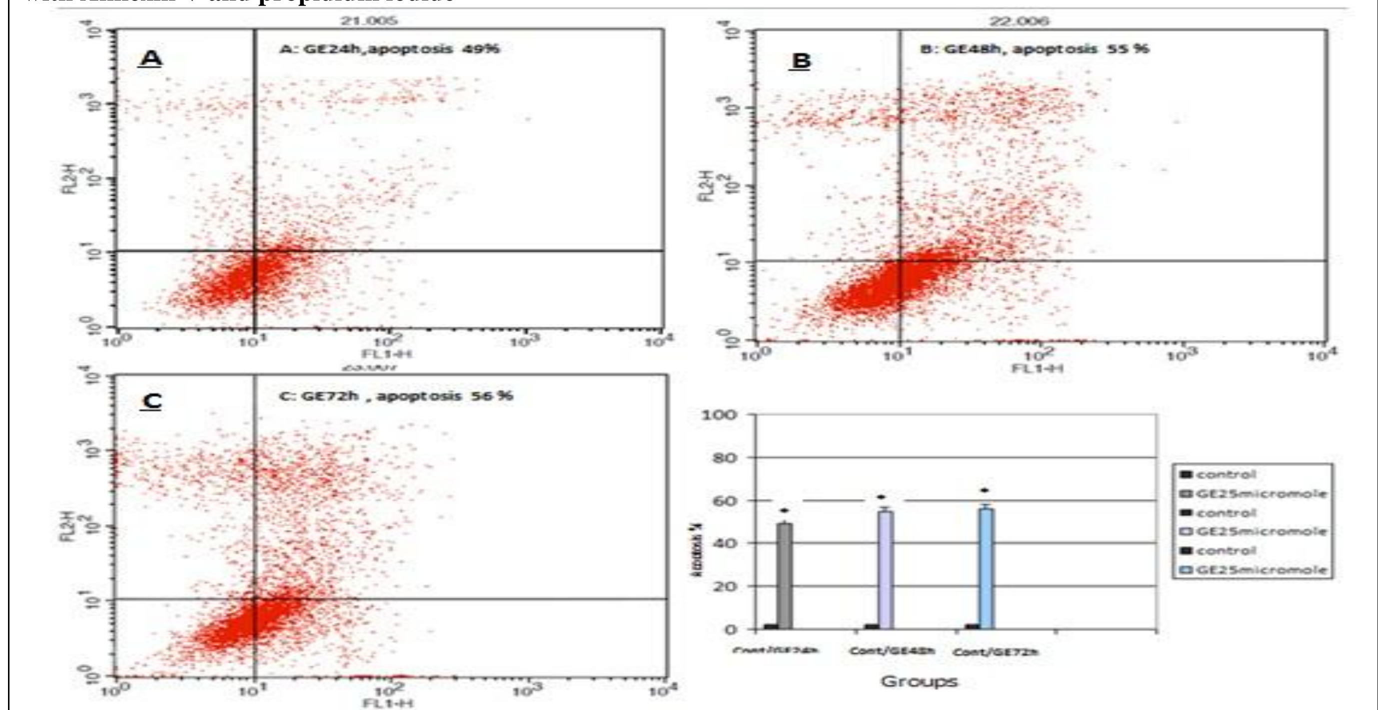


Figure 6. The apoptosis-inducing effect of TAM was investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidium iodide

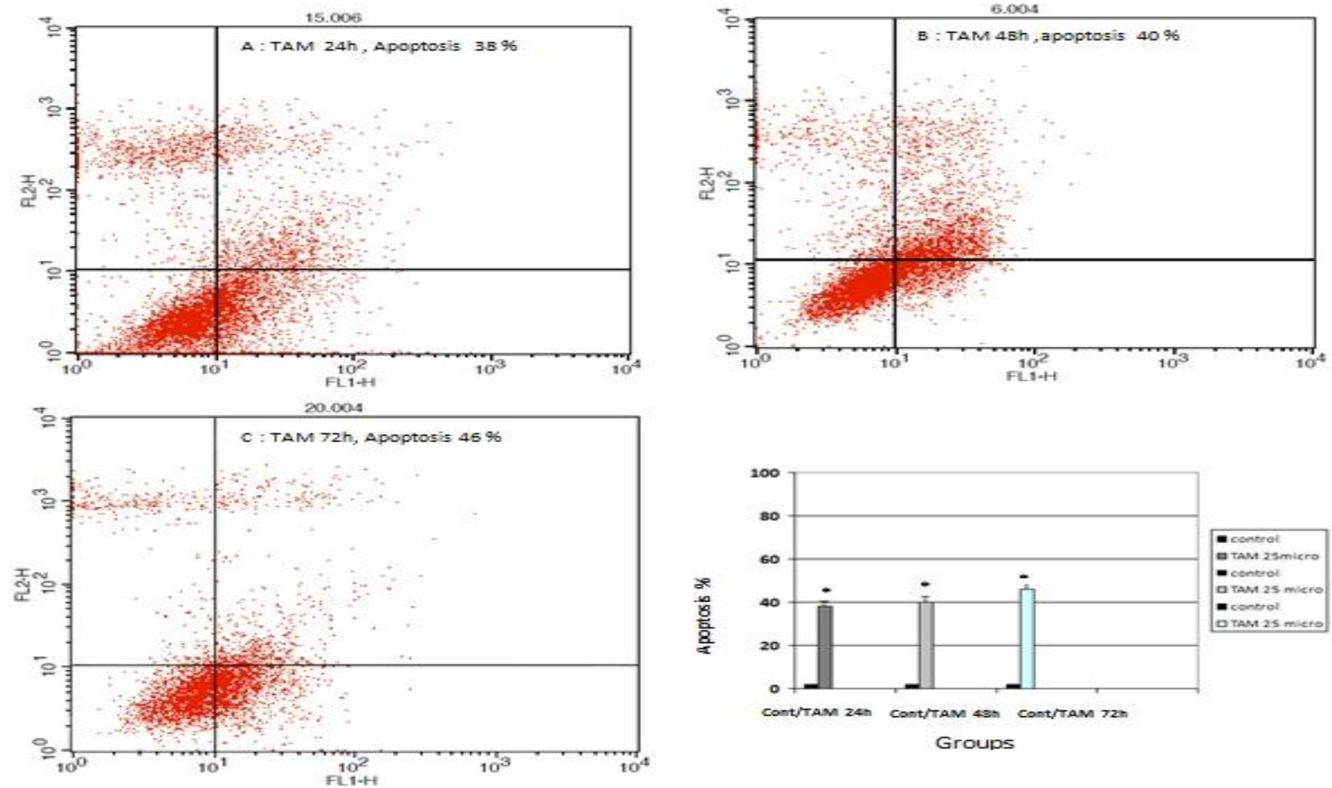


Figure 7. The apoptosis-inducing effect of GE and TAM combination (as described in section) were investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidium iodide

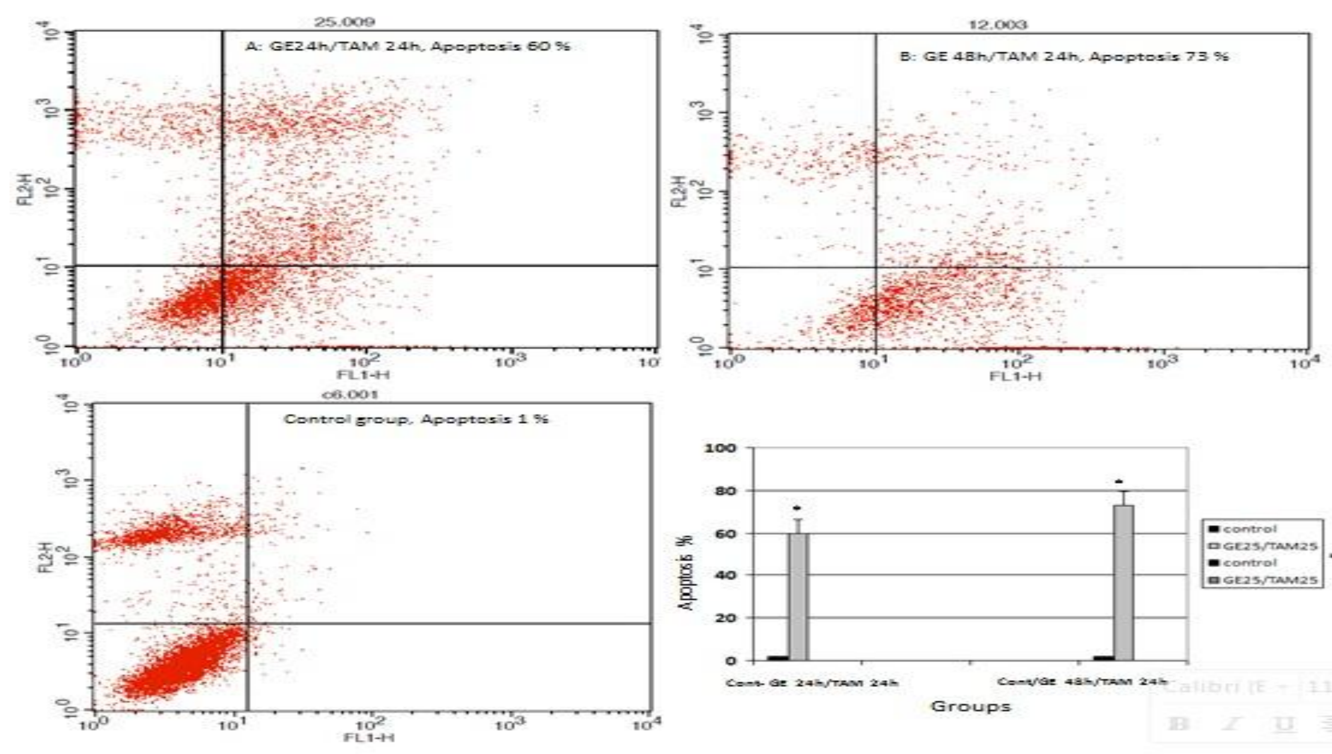


Figure 8. Relative analysis between GE treatment groups (P) and TAM treatment groups (*P) at different times indicated that GE induces apoptosis more significantly ($P < 0.001$)**

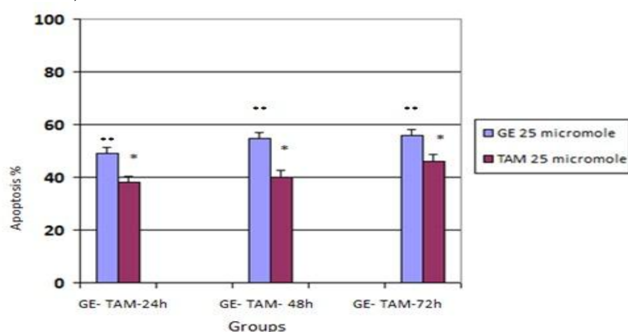


Figure 9. The percentage of apoptotic cells in the groups that treated with combined compound (GE and TAM) were significantly higher than that of the experimental groups that treated with GE or TAM alone (*$P < 0.003$, **$P < 0.001$)

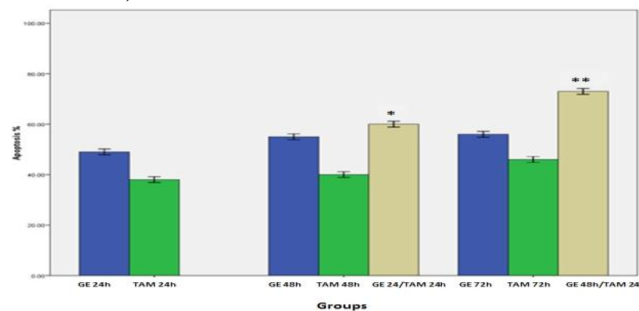


Figure 10. Time course of ERα expression in PLC/PRF5 cells in response to GE (25 µM)

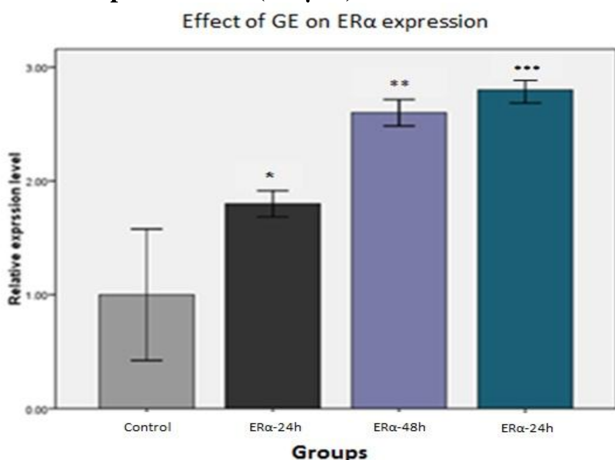


Figure 11. Time course of DNMT1 expression in PLC/PRF5 cells in response to GE (25 µM)

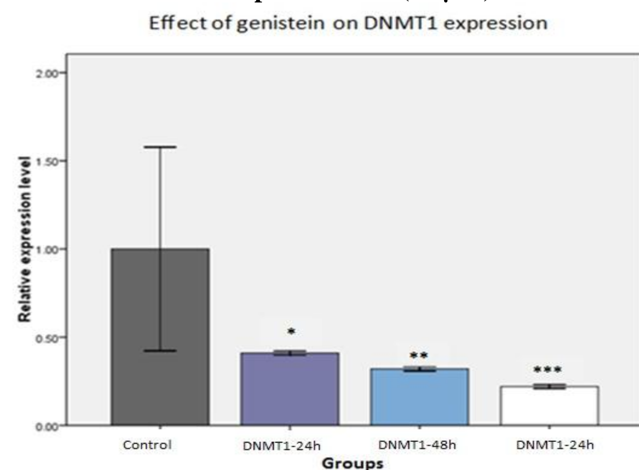
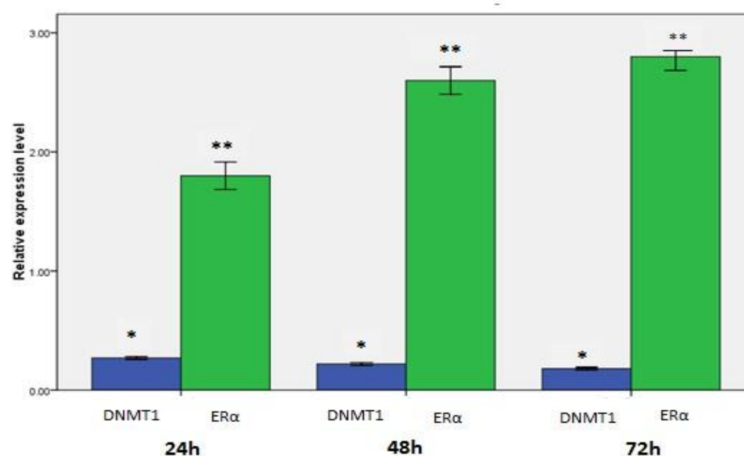


Figure 12. Relative expression level of ERα and DNMT1. GE increases ERα expression and decrease DNMT1 expression significantly



Abnormal methylation of DNA is one of the hallmarks of cancer and often leads to silence of tumor suppressor genes resulting in the development and progression of cancer. It has been shown that dietary phytochemicals play a role in epigenetic modulating and regulation of cell function [20-25]. The plant-derived polyphenolic compounds containing isoflavones have attracted a lot of attention due to anticancer properties

although their mechanism of action is not fully understood. GE inhibits growth of cancers such as colon, gastric, lung and pancreatic cancers by pleiotropic effects via the modulation of genes related to the cell cycle and apoptosis and also inhibits ovarian carcinogenesis and cancer cell growth through its pleiotropic mechanism against ER [26].

The plasma level of GE in the women consuming soy products is 0.74-6.0 µM [21] which increases antioxidant status of cells via i) interaction with estrogen receptors, ii) activation of ERK1/2 [27] and its influence most commonly is related to induction of G2/M cell cycle arrest, as shown in various type of cancers such as breast, colon, malignant glioma and prostate cancer cell lines [28,29,30], besides ,it should be noted that , its action is cell type-dependent [31,32] . Other studies have been reported that Plasma concentrations of GE is ranging from 1µM.

Our study clearly demonstrated that GE (25 µM) can down regulate the expression of DNMT1, up regulate the the expression of ERα, inhibit cell proliferation and induce cell apoptosis in PLC/PRF5 cell line with a dose- and time-dependent manner .This is consistent with other reports that have shown that GE decreases DNMT1 gene expression in other cancers [32]. Several researches have indicated that GE exerts antiprolifrative effects. Similarly, it has been reported that, GE induces and increases the apoptotic population in ovarian cancer cells. These findings about GE effect appear to lend support for our current observation but many studies have shown that GE has biphasic effect that isn't consistent with our result. They have reported that GE inhibits cell growth depending on concentration in MCF-7 cell with maximal growth stimulation at a concentration of 1µM. Martin et al and Wang et al have also reported that growth stimulation in MCF-7 cells was observed in a concentration-dependent manner between 10 nM and 1 µM. Many mechanisms and different pathways have been reported for GE; it has been demonstrated that , GE can alter cell proliferation by two dose-dependent mechanisms; one proliferative mechanism is likely to be mediated via the estrogen receptor, and antiproliferative mechanism, is likely to be mediated via anti-tyrosine phosphorylation and inhibition of cell cycle progression [6]. Similar studies have shown that, GE is a potent tyrosine kinase inhibitors and a potent modifier of epigenetic events including DNA methylation, or through steroid receptor dependent process [21]. Other studies have revealed that, GE binds to nuclear hormone receptor ER-α which undergoes conformational change and then binds to estrogen response elements and recruits coactivators, resulting in chromatin remodeling and enhancement of target gene expression [17]. Recent studies have indicated that, GE (25 µm) restores ER-α expression by remodeling the chromatin structure in the ER-α promoter in MDA-MB-231 cells [33-35]. It has been indicated that, GE induces antiprolifrative effects by modulating multiple signaling pathways such as protein-tyrosine kinase (PTK), Akt , NF-κB, matrix metalloproteinases (MMPs), and Bax/Bcl-2. It is interesting that , GE inhibits cell proliferation and induces cell cycle arrest in ovarian cancer cells by microtubule depolymerization. Furthermore, it has been shown that GE inhibits the proliferation of ovarian cancer cells by modulating the cytokine IL-6. In recent years,other studies have reported that, GE restores ER signaling via epigenetic pathways in breast cancers that do not respond to hormonal therapies [32].

According to our result, TAM inhibited proliferation and induced apoptosis and also apoptotic effect of TAM was increased when treatment with GE followed by TAM. Similar to our finding, numerous studies have shown that liver cancer, ovarian cancer, stomach cancer drug resistant, neuroblastoma and prostate cancer can be improved using TAM. Other studies have reported that, GE restores ER signaling via epigenetic pathways in breast cancers that do not respond to hormonal therapies such as TAM and also demonstrated that , GE (25µM), in particular, combined with TAM induces the maximal re expression of ER α [32]. Many studies have indicated that GE with other DNMTs inhibitor, inhibit DNMTs enzyme and reactive methylation-silenced genes [33-35].

GE can enhance the anticancer capacity of an estrogen antagonist, tamoxifen (TAM), especially in ERα-positive breast cancer cells [14]. It has been shown that, low concentration of GE reverts inhibitory effect of TAM in breast cancer [36], furthermore, it has been indicated that, the combination of GE and TAM have inhibitory effect on the growth of ER+/HER2-overexpressing BT-474 human breast cancer cells and induce apoptosis synergistically [37]. It should be noted that, in spite of our result , many studies have reported that, TAM has biphasic effects; TAM possesses agonistic and antagonistic properties depends on concentration [38]. Other investigators have demonstrated that, TAM has both estrogenic and anti-estrogenic effects that its anti-estrogenic effect is predominat .TAM can exert its effect by different mechanisms; It stimulates MCF-7 cell proliferation through a direct modulation of BK channels independent of its ER antagonist properties [39] . It acts through binding to estrogen receptors in the target cells. TAM - estrogen receptor complex binds to DNA and blocks the biological effects of intrinsic estrogen by competing with estrogen receptor. It should be noted that, TAM recruitments nuclear hormone coactivators via induction of ER serine phosphorylation and increases expression of ER-regulated growth-promoting genes and also protein kinase A, HER2/neu and Pak1implicated in ER serine phosphorylation [40-42].

The research about TAM and GE effects on HCC is rare ,herein, a research has reported that , GE (10^{-8}M) and TAM (10^{-8} M) were inhibited Hep3B cells proliferation significantly that were treated with serum-free

DMEM medium [43], therefore, we selected hepatocellular carcinoma PLC/PRF5 cell line to evaluate effects of these drugs on this cell line.

Considering the results of our research, GE and TAM maybe good candidates for HCC treatment. Treatment with GE changes DNMT1 expression *in vivo* and in vitro experiments which suggest that DNA methylation regulates transcription of ERα gene via regulation of DNMT1, therefore this mechanism should be considered in HCC treatment. In most studies, a combination of TAM and GE have been used synergistically but in our study, we first used this compounds separately and then used GE following by TAM and this is the advantage of our research compared to other researches using these compounds. We first used combination of these drugs too (data not shown) but desired results were not achieved . We did not perform enzyme activity assays related to methylation and histone modifications and also enzyme immunoassay related to protein levels that we will perform in the next researches and also further researches are needed to determine the clinical applications of GE.

CONCLUSION

Our study clearly demonstrated that GE increase ERα expression and decrease DNMT1 expression and also inhibits proliferation and induces apoptosis in human hepatocellular carcinoma cell line through epigenetic mechanism which can provide a new strategy for hepatocellular carcinoma treatment. It should be noted that, when GE (25μM) treatment followed by TAM (25μM) treatment, Apoptosis were increased more significantly.

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