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Apoptotic Effect of Genistein on Hepatocellular Carcinoma HepG 2 Cell Line

Masumeh Sanaei[1], Fraidoon Kavoosi [*1], Mostafa Arezoo [2]

1. Research center for non-communicable diseases, Jahrom University of medical sciences, Jahrom, Iran
2. Student of research committee, Jahrom University of medical sciences, Jahrom, Iran
** Corresponding Author: E-mail: kavoosifraidoon@gmail.com*

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ABSTRACT

Hepatocellular carcinoma (HCC) is a major health problem and the sixth most common cancer worldwide. Although liver cancer is the sixth most common neoplasm worldwide, it's very poor prognosis makes it the third leading cause of cancer-related mortality. This malignancy occurs more often among men than women, with the highest incidence rates reported in East Asia. GE (4',5,7-trihydroxyisoflavone), a phytoestrogenic compound and 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. The aim of the present study was to analyse the apoptotic and antiprolifrative effect of genistein in the hepatocellular carcinoma HepG2 cell line. Materials and Methods: Cells were treated with various concentration of genistein and the MTT assay was used and then cells were treated with single dose of genistein (25μM) and flow cytometry assay was performed. Results: genistein inhibited the growth of liver cancer cells and induced apoptosis significantly with a time- and dose-dependent manner. Discussion: Our finding clearly indicated that genistein has a significant inhibitory effect and induces apoptosis with a dose- and time-dependent manner. Conclusion: genistein can significantly inhibit the growth of HCC cells and plays a significant role in apoptosis of this cell line.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem and the sixth most common cancer worldwide (1). The incidence of HCC is increasing in Europe and in the US (2) and it is a leading cause of death among cirrhotic patients(3). Although liver cancer is the sixth most common neoplasm worldwide, it's very poor prognosis makes it the third leading cause of cancer-related mortality, responsible for 600,000 deaths annually (4). In most countries, HCC accounts for 70%–85% of primary liver cancer cases (5), with the burden of disease expected to increase in coming years (6).

This malignancy occurs more often among men than women, with the highest incidence rates reported in East Asia (7).The incidence rates of HCC in the United States have historically been lower than in many countries. However, in recent decades, HCC age-adjusted incidence rates have doubled (8) and primary liver cancer mortality rates have increased faster than mortality rates for any other leading cause of cancer (9, 10). Approximately 90% of primary liver cancers in the United States are HCCs, while most of the remaining 10% are intrahepatic cholangiocarcinomas (11).The pathway leading to HCC generally begins with an acute hepatic insult which progresses over decades. Fibrosis and cirrhosis are typically precursors of HCC (12).

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 (13). 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 (14) that change gene expression and cell function (15).

Genistein (GE), a phytoestrogenic compound and a major isoflavone constituent of soybeans and soy products, has been shown to suppress the growth of various cancers such as breast, ovarian, oesophagus, lung and colon cancers through modulation of various pathways. GE has multiple molecular targets including receptors, enzymes, and signaling pathways (16). Many studies have indicated that the incidence and mortality rates of these cancers are

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considerably lower in Asia compared to the United States because of consumption of soybeans and soy products (17). Phytoestrogens, originating from various plant sources include fruits, vegetables, legumes, soy beans, whole rye and flax seeds and whole grains. Infact, phytoestrogens are phenolic non-steroidal plant derived compounds possessing estrogen like activity, the structure of which is similar tothat of 17-β-estradiol (18). This group is divided into three subgroups: isoflavones, lignans, and coumestans which the lignans are usually broken down to enterolactone and enterodiol before absorbtion (19). Generally, phytoestrogens are ingested as conjugates and then reconjugated in the liver and intestinal epithelium by glucuronosyl and sulphotransferases (20).

There is a good correlation between hormonal activity and carcinogenicity of estrogen (21-23). Recent data indicates that phytoestrogens decrease tumor progression in HCC in vivo by reducing tumor cell invasion, arresting cell cycle progression, and promoting apoptosis (24) and also estrogen and the estrogen-like compounds may induce antiproliferative and apoptotic effects in Hep3B cells (25).

Since there is few studies for genistein effect on HepG2 and we cannot find any data about effect of genistein on HepG2 cell line (in vitro),therefore ,this study was designed to investigative the apoptotic and anti-proliferative effects of genistein on the human hepatocellular HepG2 cell line.

MATERIALS AND METHODS

Human hepatocellular carcinoma cells (HepG2) were purchased from the National Cell Bank of Iran-Pasteur Institute. Genistein, DMEM (Dulbecco minimal essential medium) 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 sources available.

CELL CULTURE

The cells were cultured in DMEM with pH 7.2–7.4 (Sigma) containing 1% sodium pyruvate (sigma), 3.7 mg/ml sodium bicarbonate (Sigma), 10% fetal bovine serum (FBS) (sigma) and 1% antibiotics which include 10,000 units/ml penicillin G sodium (sigma), 10,000 ug/ml streptomycin sulfate and 25 ug/ml amphotericin B (sigma) at 37°C in 5% CO2 to promote attachment. When cells became >80% confluent, 5×10^5 cells were seeded into 24-well plates (Becton-Dickinson) for 24 h in DMEM culture medium before they were incubated with certain concentrations of genistein (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 based on IC50 index, at different times (24, 48 and 72 h). The control cells were treated with DMSO only. Photography was done for cultures before and after treatment with genistein at different times using inverted microscope (Nikon, TE 2000-U, Japan).

DETERMINATION OF IC50 VALUE BY MTT ASSAY

The effect of genistein on cellular proliferation was assessed by MTT assay, according to standard protocols. After 24, 48 and 72 h of the treatment, the IC50 value for genistein in HepG2 groups were determined. The MTT assay was commonly used to assess cell proliferation and viability by measuring of the reduction of yellow MTT by mitochondrial dehydrogenases in viable cells. Briefly, 5×10^5 Cells were counted and placed into each well of a 24-well micro plate and were treated with various drug concentrations (1, 5,10, 25, 50, 75, and 100 μM/lit) of genistein for 24 ,48 and 72h and the MTT survival assay was then carried out for the evaluation of the cell viability with different drug concentrations. The cells were measured spectrophotometerically at 540 nm. All experiments were repeated three times, with at least three measurements (triplicates).

DETERMINATION OF CELL VIABILITY BY MTT ASSAY

To determine the effect of genistein, the cells were seeded in triplicate in 24-well plates and treated with genistein at concentration of 25 μM in different period times (24, 48 and 72h).The cell viability was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product . The absorbance of the cell lysates in DMSO solution was read at 540 nm by a microplate reader (Bio-Rad Hercules, CA).

DETERMINATION OF APOPTOTIC CELLS BY FLOW CYTOMETRY ASSAY

The cells were seeded in 24-well plates. After 24 h, the medium was changed and medium contain genistein (25μM) was added. After 24 ,48 and 72 h of incubation, all the adherent cells were collected with 0.05% trypsin, washed with cold phosphate-buffered saline (PBS) and resuspended 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 FACScan 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.

RESULT OF DETERMINATION OF CELL VIABILITY BY MTT ASSAY

The cell vitality in the cells treated with genistein at concentration of 25 μM in different time periods were analysed by using the MTT assay. The amounts of reduced MTT in the all groups treated with genistein were significantly lower than that of the control group ($P < 0.001$). The percentage of living cells in treatment groups at different time

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periods (24, 48 and 72h) were 57, 48 and 41%, respectively at a concentration of 25 μM of genistein. This experiment was repeated three times for each group (fig.1).

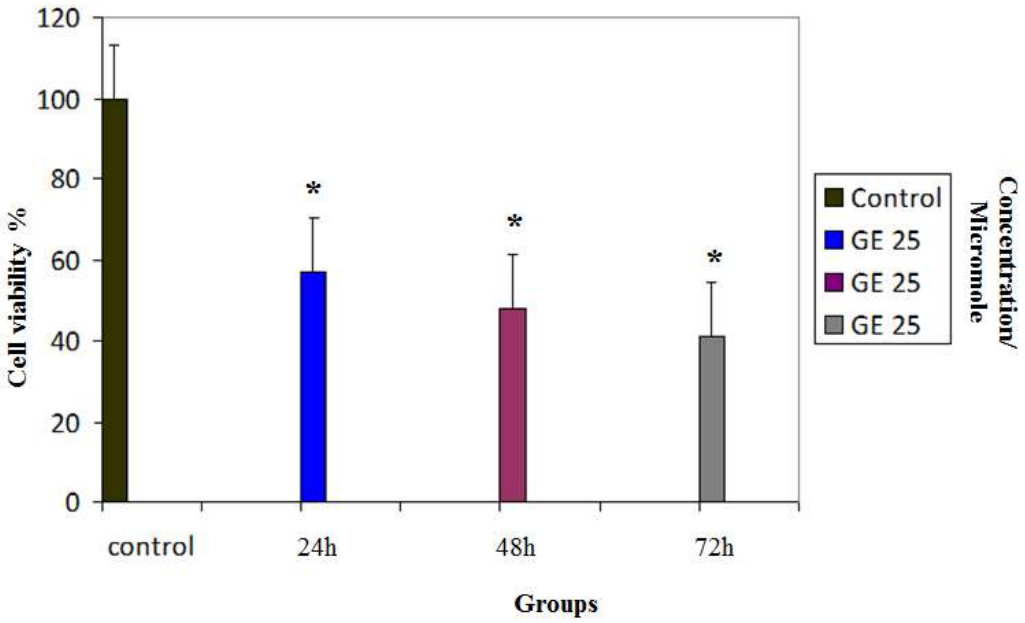

Fig 1. Effect of GE on cell viability of HepG2 cells. The effect of GE on the viability of HepG2 cells was determined by MTT assay at different time periods (24, 48 and 72h). Mean values from the three experiments± standard error (S.E.M) are shown. Asterisks indicate significant differences between treated cells and the control group. P<0.001

RESULT OF DETERMINATION OF APOPTOTIC CELLS BY FLOW CYTOMETRY ASSAY

The cells were treated with 25 μM concentration of genistein for different time periods (24, 48 and 72h). Flow cytometry was performed to observe the apoptotic cells which had been visualized using Annexin V-FITC and/or propidium iodide staining. Flow cytometric analysis indicated that genistein at 25 μM concentration induces apoptosis in hepatocellular cancer cells in a time – dependent manner ($P < 0.001$). The amount of apoptotic cells was significantly increased in all three groups. Percentage of apoptotic cells at different time periods (24, 48 and 72 hours) were 38, 59 and 62%, respectively. Apoptotic effects were not observed in DMSO group (Fig.2 and 3).

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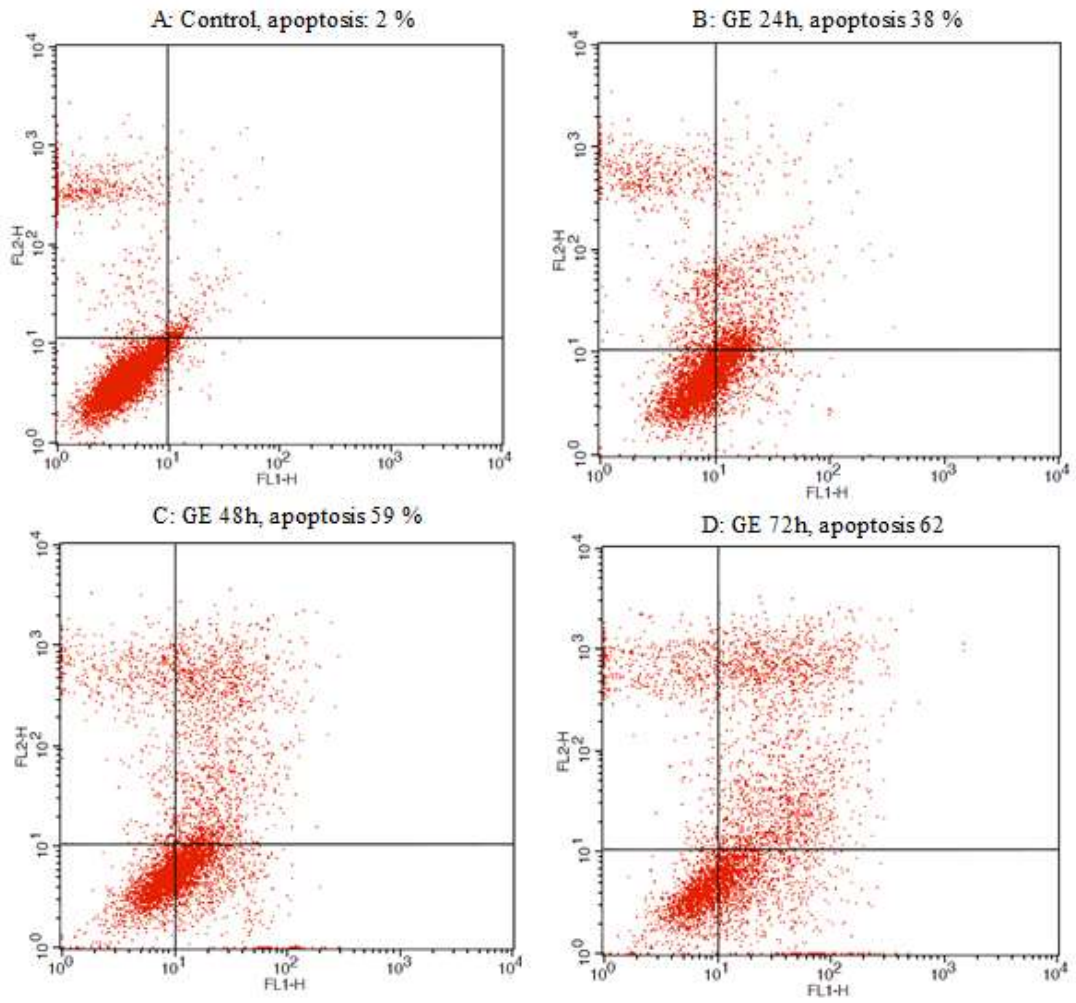

Fig2. Effect of GE on HepG2 cells apoptosis. The cells were treated with GE (25 μM) for 24, 48 and 72h and the apoptosis- inducing effect of GE was investigated by flow cytometric analysis of HepG2 cells stained with Annexin V and propidium iodids.

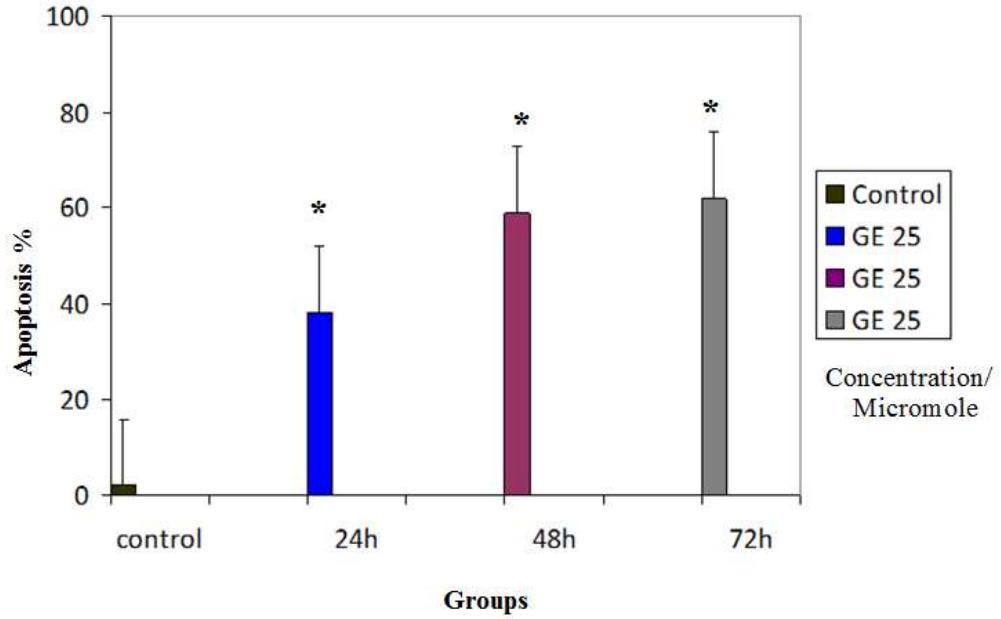

Fig3. Apoptotic-inducing effects of GE (25µM) for 24, 48 and 72h. Result were obtained from three independent experiments and were expressed as mean± standard error of mean (S.E.M). Asterisks indicate significant differences between treated cells and the control group. P< 0.001, n=3, A: control, B: GE 24h, C: GE 48h, D: GE 72h

DISCUSSION

Hepatocellular carcinoma (HCC) is a malignancy of worldwide significance. It is currently the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death (26). Eighty percent of new cases occur in developing countries, but the incidence is increasing in economically developed regions, including Japan, Western Europe, and the United States (27, 28). Liver cirrhosis is the seventh leading cause of death in the world and the 10th most common cause of death in the United States, and is acknowledged as a premalignant condition for developing HCC (29, 30). In the United States, hepatitis C virus (HCV), alcohol use, and nonalcoholic fatty liver disease are the most common causes of cirrhosis (31).

Numerous epidemiological studies suggest that diets rich in phytoestrogens (PE), particularly soy and unrefined grain products, may be associated with low risk of some cancers, especially steroid hormonedependent, *e.g.* breast and prostate cancers (32, 33). Many *in vitro* experiments detected anticancer effects of PE at high concentrations (but mild stimulatory effects at lower concentrations). Animal studies have revealed both cancerinhibitory and cancer-promoting effects. It has been shown

that genistein (GEN, an isoflavonoid present in soy beans) may inhibit *in vitro* human and mouse mammary cancer cell proliferation and invasion (34-36). However, this effect can be achieved only in high doses, ranging from 10 to 100 M, while at lower concentrations it may stimulate the proliferation of estrogen-dependent tumor cells. However, GEN, like other estrogen agonists, may exert its chemopreventive activity by enhancing mammary cell maturation, thus reducing cell proliferation (37-39).

Our data clearly shown that genistein has a significant inhibitory effect on the growth of liver cancer cells and induces apoptosis in this cell line with a time-dependent manner.

This is consistent with other reports that have shown that genistein inhibits the growth of MDAMB-231 breast cancer and induces apoptosis through a p53-independent pathway. Genistein was found to inhibit MDA-MB-435 and 435.eB cell growth. Induction of apoptosis was also observed in these cell lines when treated with genistein. Thus, genistein inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis and may inhibit invasion and metastasis (40).

It has reported that dietary genistein inhibits prostate cancer metastasis and that genistein inhibits initial steps in the metastatic cascade, namely, cell detachment and cell invasion (41-44). Epidemiologic findings further support the notion that genistein may be active in man. A number of studies associate dietary consumption of genistein with a lower mortality from prostate cancer, and of particular interest, some suggest that there may be little effect upon the incidence of organ-confined prostate cancer (45–48). In addition to the epidemiological evidence, several biological mechanisms through which soy phytoestrogen reduces the risk of prostate cancer have been proposed, including inhibition of angiogenesis (49), inhibition or stimulation of regulatory proteins in the cell cycle (50), and inhibition of signal transduction pathway involving epidermal growth factor (51). Genistein is now considered to be the primary anticancer component of soybeans; it s in vitro and/or in vivo activities include the antagonism of estrogen, inhibition of protein tyrosine phosphorylation, suppression of angiogenesis, inhibition of hydrogen peroxide formation induced by tumor promoters, inhibition of topoisomerases, induction of apoptosis and cell differentiation, scavenging of free radicals, and inhibition of carcinogenesis and tumor promotion. In animal models of mammary gland, liver, colon,

skin, prostate, and stomach carcinogenesis, soy consumption reduces the development of cancers (52-58). Dietary genistein reduces the multiplicity of mammary and prostate tumors that develop in carcinogen-dosed murine models (59-60). In mammary tumors, the protective effects are seen for both estrogen-dependent and -independent tumors. Dietary genistein also reduces the incidence of aberrant crypts and colon cancer in carcinogen-dosed rats (61). In addition to its cancer preventive effects, genistein has a role in cancer therapy (62-63).

These works confirm our data obviously, but many studies have shown proliferative effects of GE that is opposite of our finding; it has been reported that GE induces apoptosis in the prostatic cancer LAPC‐4 cells but has biphasic effect in the LNCap cell line. In fact, this compound has proliferative effect with physiological concentration (<10 μM) and inhibitory response with high concentration (25 or more than 25 μM) (64). Similar studies also have shown that a low dose of GE (3.7 μM) has a proliferative effect on human intestinal cells and inhibitory effect with 26-111 μM concentration. Moreover, it stimulates cell growth with <3.7 μM concentration in IEC18 cell line(65) and also stimulates cell growth in the ER‐positive MCF‐7 breast carcinoma cells with concentrations of 1 nM to 10 μM/L (66).

CONCLUSION

Our findings suggest that, genistein may be a potent antiestrogenic compound and can effectively inhibit growth and induce apoptosis in HepG2 HCC cells. In future studies, the mechanisms and pathways of antiestrogenic effects of genistein on HepG2 should be evaluated.

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