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Stimulatory and Inhibitory Effects of Low and High Concentrations of Genistein on Human Hepatocellular Carcinoma HepG2 Cell Line

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. It is a fatal disease with a high incidence in sub-Saharan Africa, China, and the Far East and a low incidence in the United States and Europe. The major recognized risk factors for the disease are viral, toxic, metabolic and immune-related. Soy isoflavones have been identified as dietary components having an important role in reducing the incidence of cancers. Genistein (GE), the predominant isoflavone found in soy products, has biphasic effects. It has stimulatory or inhibitory effects according to concentration. The aim of the present study was to analyze the stimulatory and inhibitory effects of genistein on hepatocellular carcinoma HepG2 cell line. Materials and Methods: Cells were treated with various concentrations of genistein and the MTT assay was used and then cells were treated with single dose of genistein (20μM) and flow cytometry assay was performed. Results: genistein indicated stimulatory and inhibitory effects according to concentration. Discussion: Our finding clearly indicated that genistein has a significant inhibitory and stimulatory effects related to concentration. Conclusion: genistein can play biphasic role on the growth of HCC cells.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. It is a fatal disease with a high incidence in sub-Saharan Africa, China, and the Far East and a low incidence in the United States and Europe.[1]. Incidence of disease is increasing and it has risen to become the 5th commonest malignancy worldwide and the third leading cause of cancer related death [2]. The estimated incidence of new cases is about 500 000-1 000 000 per year, causing 600 000 deaths globally per year [3-7]. The major risk factor of HCC is cirrhosis of the liver. However, about one quarter of HCC cases diagnosed in the United States do not have any known predisposing risk factors. The major recognized risk factors for the disease are viral (chronic hepatitis B and hepatitis C), toxic (alcohol and aflatoxins), metabolic (diabetes and non-alcoholic fatty liver disease, hereditary haemochromatosis) and immune-related (primary biliary cirrhosis and autoimmune hepatitis) [8].
Clinical methods such as hepatic resection, percutaneous ethanol injection, and transcatheter arterial embolization have all been used in the treatment of patients with small-sized hepatocellular carcinomas [9]. These methods are very expensive and very recurrent. Epigenetic drugs and treatment is a new and effective method for cancer therapy. Epigenetic mutations, affect a variety of cancer-related genes. Epigenetic silencing of tumor suppressor genes is associated with promoter hypermethylation [10-11]. It has long been known that DNA methyltransferase inhibitors can effectively induce DNA demethylation and phenotypic changes related to the reactivation of epigenetically silenced genes [12]. These findings were later adapted to the targeting of epigenetic mutations in cancer and thus established the fundamental concept of epigenetic cancer therapy [13].
Epigenetic mechanisms controlling gene transcription are often involved in cell proliferation, differentiation, and survival and are casually linked with malignant development. Alterations in epigenetic processes including chromatin modifications such as DNA methylation and histone acetylation are common targets studied in cancer epigenomics [14-15]. It has been shown that half of all tumor suppressor genes are inactivated in cancers more often by epigenetic, than by genetic, mechanisms [16]. Growing evidence suggests that bioactive dietary components

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impact epigenetic processes often involved with reactivation of tumor suppressor genes, activation of cell survival proteins, and induction of cellular apoptosis in many types of cancer [17-18]. Soy isoflavones have been identified as dietary components having an important role in reducing the incidence of cancers. Genistein (GE), the predominant isoflavone found in soy products, has been shown to inhibit the carcinogenesis in animal models. There is a growing body of experimental evidence showing that the inhibition of human cancer cell growth by genistein is mediated via the modulation of genes that are related to the control of cell cycle and apoptosis [19].
Our previous work indicated that genistein (25μM) significantly inhibits the growth of HCC HepG2 cells and plays a significant role in apoptosis of this cell line [20].
Since there is few studies for effect of biphasic effects of genistein on HepG2 and we cannot find any data about the biphasic effects of genistein on HepG2 cell line (in vitro), therefore, this study was designed to investigative the stimulatory and inhibitory effects of genistein on HCC HepG2 cell line.

METHODS

Human HCC HepG 2 cells were purchased from the National Cell Bank of Iran‐Pasteur Institute. GE, Dulbecco minimal essential medium (DMEM) and MTT (3‐[4,5‐dimethyl‐2‐thiazolyl]‐2,5‐diphenyl ‐2H‐tetrazolium bromide) were purchased from Sigma (Sigma, St. Louis, MO, USA). All other chemicals were obtained from the best sources available.

Cell Culture

The HepG 2 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 (sigma) and 1% antibiotics which include 10,000 units/ml penicillin G sodium (sigma), 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B (sigma) at 37°C in 5% CO2 to promote attachment. When the 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 GE (0.5, 1, 5, 10, 20 μM/L), which was dissolved in dimethyl sulfoxide (DMSO); DMSO was present at 0.01-0.3% in the medium based on IC50 index, at different time periods (24, 48). The control cells were treated with DMSO only. Photography was done for cultures before and after treatment with GE at different time periods using inverted microscope (Nikon, TE 2000‐U, Japan).

Determination of IC50 value by MTT assay

The effect of GE on cellular proliferation was assessed by MTT assay according to standard protocols. After 24 and 48 h of the treatment, the IC50 value for GE were determined. 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. Briefly, 5×10^5 Cells were counted and placed into each well of a 24‐well microplate and were treated with various drug concentrations (0.5, 1, 5, 10, 20 μM/L) of GE for 24 and 48 h and the MTT survival assay was then carried out for the evaluation of the cell viability with different drug concentrations. The cells were measured spectrophotometrically at 570 nm. All experiments were repeated 3 times, with at least three measurements (triplicates).

Determination of cell viability by MTT assay

To determine the effect of GE, the cells were seeded in triplicate in 24‐well plates and treated with GE at a concentration of 20 μM/L in different period times (24 and 48 h). 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 570 nm by a microplate reader (Bio‐Rad Hercules, CA, USA).

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 contains GE (20 μM/L) was added. After 24 and 48 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 (×1). After addition of Annexin V‐FITC and propidium iodide (PI, Becton‐Dickinson, San Diego, CA, USA), 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.

RESULTS

Result of determination of IC50 by MTT assay

Cell vitality in the human HCC cell line was analyzed using the MTT assay as described previously. The result of MTT assay indicated that GE inhibits the growth of liver cancer cells significantly with concentration of 5, 10, 15 and 20 μM/L and enhances proliferation with concentration of 0.5 and 1 μM/L. The IC50s value for HepG 2 cells were 20 μM/L of GE at different time periods (24 and 48 h). The effect of GE was dose‐ and time‐dependent. This experiment was repeated three times for each group.

Result of determination of cell viability by MTT assay

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The cell vitality in the cells treated with GE at a concentration of 20 µM/L in different time periods was analyzed using the MTT assay. 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 living cells in treatment groups (24 and 48 h) were 56% and 47%, respectively, at a concentration of 20 µM/L of GE. This experiment was repeated three times for each group (Figure 1).

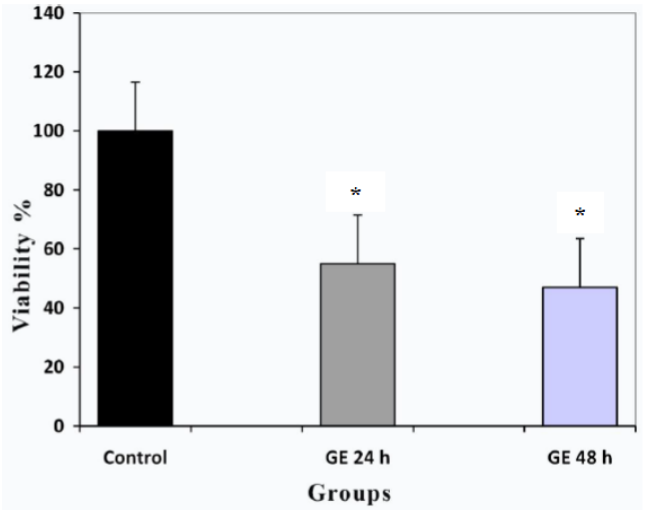

Fig 1. The effect of GE (20 µM/L on cell viability of HepG2 cells. The effect of GE on the viability of HepG2 cells was determined by MTT assay at different time periods (24 and 48 h). Mean values from the three experiments± standard error (S.E.M) are shown. Asterisks indicate significant differences between treated cells and the control group. $P<0.001$

Result of determination of apoptotic cells by flow cytometry

The cells were treated with 20 µM/L concentration of GE for different times (24 and 48 h). Flow cytometry was performed to observe the apoptotic cells which had been visualized using Annexin V‑FITC and/or PI staining. Flow cytometry analysis indicated that GE at 20 µM/L concentration induces apoptosis in hepatocellular cancer cells in a time‑dependent manner. The amount of apoptotic cells was significantly increased in experimental groups, but an apoptotic cell in the 48 h treatment group was more significant [Figure 3]. Percentage of apoptotic cells at different time periods (24 and 48 h) were 34, 44 % respectively. Apoptotic effects were not observed in DMSO group (Figure 2)..

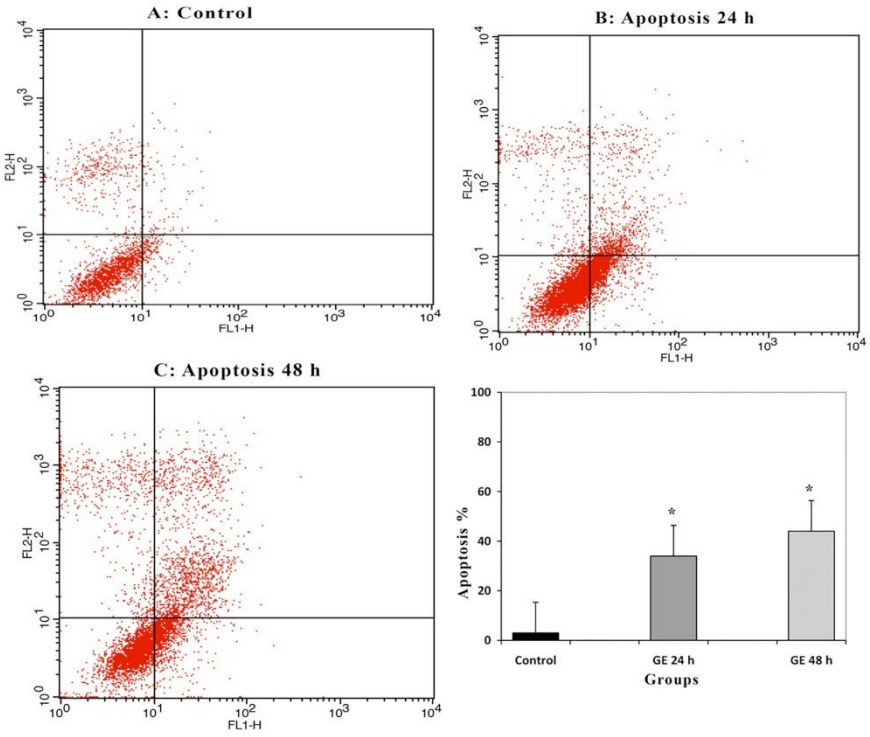

Fig2. Effect of GE with concentration of 20 μM/L on HepG2 cells apoptosis. The cells were treated with GE (20 μM) for 24 and 48 h and the apoptosis- inducing effect of GE was investigated by flow cytometric analysis of HepG2 cells stained with Annexin V and propidium iodids

DISCUSSION

Epigenetic mechanisms are required to maintain normal growth and development and gene expression in different organs (21). Abnormal epigenetic regulation may alter gene expression and function which may lead to diseases such as cancer. Human tumors, in essence, are a genetic disease, since during cancer formation, a large number of genes are mutated or abnormally activated. However, recent studies indicate that carcinogenesis cannot be accounted for by genetic alterations alone, but also involve epigenetic changes such as DNA methylation, histone modifications and microRNAs (22-23). Epigenetic defects in cancer cells can be efficiently reverted by means of pharmacologic inhibitors of the enzymes that are responsible for establishing/maintaining the epigenetic marks (24). Epigenetic therapy tries to reverse the aberrations followed to the disruption of the balance of the epigenetic signalling ways through the use of both natural compounds and synthetic molecules, active on specific epi-targets. Flavonoids are polyphenolic phytochemicals that exert a multitude of beneficial effects on human health. In recent years, isoflavones, flavonols and catechins have received much attention due to their ability to influence activity of chromatin-modifying enzymes (25). Genistein, one of the predominant soy isoflavones causes inhibition of cancer cell growth in vivo and in vitro. It can regulate the genes that are critical for the control of cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, and cell signal transduction pathways (26).
Our study clearly indicated that GE has a biphasic effect. It had a significant prolifratory effect with low concentration and a significant inhibitory and apoptotic effect with high concentration on the liver cancer cells. Our previous study indicated that GE (with concentration of 25 μM/L) has a significant inhibitory effect on the growth of PLC/PRF5 hepatocellular carcinoma cell line and induces apoptosis in this cell line with a dose‐ and time‐dependent manner (27). Previously we demonstrated that combination of GE and E2 induces apoptosis and inhibits proliferation more significant than that of these compounds alone (28) and also genistein has a significant inhibitory effect on the growth of liver cancer HepG 2 cells and induces apoptosis in this cell line with a time-dependent manner (29). Similarly, the same conclusion was reached by other researchers in other cancers; Chang KL et al., reported that GE inhibits proliferation and induces apoptosis in human prostate cancer (30) and also isoflavones inhibit growth of HT‐29 and colo320 cell. Furthermore, other investigators reported that GE inhibits the growth of HCT 116 cells with a dose‐dependent manner. This compound inhibits the growth of breast cancer cell lines ADA/MB231, MCF‐7 and HBL‐100 too (31). Similar to our work it has demonstrated that genistein has proliferative effect on human WRO, FRO, and ARO thyroid carcinoma cells and induces breast and uterus cancer development (32-33). Collectively, GE induces significant growth with a low concentration and significant inhibition with a high concentration.

CONCLUSION

Our findings suggest that, genistein has biphasic effects with an inhibitory and apoptotic effect with a high concentration and with a proliferative effect with a low concentration on HepG2 HCC cells. In future studies, the mechanisms and pathways of antiestrogenic effects of genistein on HepG2 should be evaluated.

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