Hepatocellular carcinoma (HCC) is one of the major malignant diseases in the world today. Phytoestrogens are a group of natural compound with estrogen-like activity. The aim of the present study was to analyse the apoptotic and antiproliferative effect of 17-beta estradiol in the hepatocellular carcinoma PLC/PRF5 cell line. Materials and Methods: Cells were treated with various concentration of E2 and the MTT assay was used. Furthermore, Cells were treated with single dose of E2 (25µM) and flow cytometry assay was performed. Results: E2 inhibited the growth of liver cancer cells and induced apoptosis significantly with a time- and dose-dependent manner. Discussion: Our finding clearly indicated that E2 has a significant inhibitory effect and induces apoptosis with a dose- and time-dependent manner. Conclusion: E2 can significantly inhibit the growth of HCC cells and plays a significant role in apoptosis of this cell line.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major malignant diseases in the world today and has increased in numbers year by year. The disease is a common malignancy in developing countries and the fifth most common cancer in men and the seventh in women and also the third most common cause of cancer-related deaths worldwide (1). The geographical prevalence of HCC isn’t uniform in different regions of the world. More than 700,000 new cases are diagnosed each year throughout the world and also unfortunately more than 600,000 deaths are attributed to HCC each year. The distribution of the disease is related to distribution of HBV and HCV infection, with the highest rates of HCC in regions with high infection, the major risk factors of HCC (2-5).

The incidence of HCC in Asian countries is lower than that of the developing countries (6-9). Major risk factors of HCC including; chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV), cirrhosis, alcohol consumption, dietary digestions of aflatoxin (Aflatoxin is a mycotoxin that commonly contaminates corn, soybeans, and peanuts), obesity, diabetes mellitus, iron overload and tobacco smoking (10). Other risk factors include male gender, advanced age (11), hereditary hemochromatosis, environmental toxins, oral contraceptive. It should be noted that, HCC can also occur without known risk factors (12,13).

Phytoestrogens are a group of natural compound with estrogen-like activity and similar structure of estradiol, biologically active phenolic compounds that structurally mimic the mammalian estrogen 17-betaestradiol(E2). 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 to that of 17-betaestradiol (fig.1) (14). This group is divided into three sub groups: isoflavones, lignans, and coumestans which the lignans are usually broken down to enterolactone and enterodiol before absorption (15).

Phytoestrogens are hydrolysed in the gastrointestinal tract with the help of bacteria before entering the blood stream and also lignans secoisolariciresinol and matairesinol are changed to mammalian lignans enterolactone and enterodiol by human gut bacteria (16). Generally, phytoestrogens are ingested as conjugates and then reconjugated in the liver and intestinal epithelium by glucuronosyl and sulphotransferases (17).

Fig.1 about here

Phytoestrogens are known to act as agonists or antagonists of E2 (18). They exert weak estrogenic activity in some tissues and antiestrogenic activity in others. Really they exert dual actions (both inhibitory and stimulatory effects) depending on their concentration (19-21) and have both tumorigenic and antitumorigenic effects. Because of anti-proliferative effect, they protect against some cancer such as, uterine, breast, prostate, lung and colon cancer (22-25). Therefore, there is an ever growing interest in treatment with these compounds. High consumption of phytoestrogens is associated with low incidence of cancer risk. The incidence of breast cancer in Asian populations that consume large amount of compound rich in phytoestrogen is lower than Western populations with low intake of these compounds (26-29). Many epidemiologic studies indicated an association between high consumption of foods rich in phytoestrogens and low incidence of prostate cancer (30).

Similar epidemiologic studies demonstrated that high intake of soy product (main source of phytoestrogen) reduces colorectal cancer risk (31-36) and also increasing intake of soy foods is associated with a reduced risk of lung cancer.

It has been shown that phytoestrogens have anti-metastatic and anti-angiogenic effects and play a role in cell cycle arrest. Furthermore, phytoestrogens have significant inhibition on distant metastases of breast, prostate, bladder, and pancreatic cancer in animal models (37). 17-β-estradiol can act as a carcinogenic agent and is able to induce complete neoplastic transformation of human breast epithelial cells (38-44). On the other hand, studies of breast cancer
have demonstrated that estrogen is a mammary-gland carcinogen(45). It is also known that renal carcinoma can be induced by exposure to estrogens (46). There is a good correlation between hormonal activity and carcinogenicity of estrogen in the hamster kidney (47-49). Recent data indicates that estrogens decrease tumor progression in HCC in vivo by reducing tumor cell invasion, arresting cell cycle progression, and promoting apoptosis(50) and also estrogen and the estrogen-like compounds may induce anti-proliferative and apoptotic effects in Hep3B cells(51).

Since there is few studies for E2 effect on HCC and we can’t find any data about effect of E2 on PLC/PRF5 cell line (in vitro), therefore, this study was designed to investigate the apoptotic and anti-proliferative effects of E2 on the human hepatocellular PLC/PRF5 cell line.

MATERIALS AND METHODS

Human hepatocellular carcinoma cells (PLC/PRF5) were purchased from the National Cell Bank of Iran Pasteur Institute, E2, DMEM (Dulbecco minimal essential medium) and MT (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 1% antibiotics which include 10,000 units/ml penicillin G sodium (Sigma, St. Louis, MO) .

RESULTS

RESULT OF DETERMINATION OF IC50 BY MTT ASSAY

Cell viability in the cells treated with E2 at concentration of 25 μM in different time periods were analysed by using the MTT assay. The amounts of reduced MTT in all treatment groups except the control group (P < 0.001) were incubated with certain concentrations of E2 (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.

DETERMINATION OF IC50 VALUE BY MTT ASSAY

The effect of E2 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 E2 in PLC/PRF5 groups 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 x 10^4 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 E2 for 24, 48 and 72 h and the MTT survival assay was then carried out for the evaluation of the cell viability with different drug concentrations.

DISCUSSION

Hepatocellular carcinoma represents approximately 4% of liver cancer cells in a time - dependent manner (P < 0.001). The percentage of living cells in treatment groups (24, 48 and 72h) at a concentration of 25 μM were 51%, 48%, 45 %, respectively (P < 0.001). The effect of E2 was dose- and time-dependent.

The cells were treated with 25 μM concentration of E2 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 E2 at 25 μM concentration induces apoptosis in hepatocellular cancer cells in a time – dependence manner (P < 0.001). The amount of apoptotic cells was significantly increased in all three groups (Fig.4). Percentage of apoptotic cells at different time periods (24,48 and 72 hours) were 28, 36 and 62%, respectively. Apoptotic effects were not observed in DMSO group.

DETERMINATION OF APOPTOTIC CELLS BY FLOW CYTOMETRY ASSAY

The cells were treated with 25 µM concentration of E2 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 E2 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 (Fig.4). Percentage of apoptotic cells at different time periods (24,48 and 72 hours) were 28, 36 and 62%, respectively. Apoptotic effects were not observed in DMSO group.

Fig. 3 about here

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Fig. 4 about here

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all new cancer cases diagnosed worldwide. Main risk factors of HCC including; HBV, HCV. Other risk factors include cirrhosis, alcohol consumption, dietary digestions of aflatoxin (Aflatoxin is a mycotoxin that commonly contaminates corn, soybeans, and peanuts), obesity, diabetes mellitus, iron overload and tobacco smoking(52). Phytoestrogens display pharmacologic properties with both tumorigenic and anti-tumorigenic effects. Epidemiologic evidence have indicated that diet and nutrition rich in phytoestrogen can influence cancer development and high intake of phytoestrogens reduce various cancers(53).

Our data clearly shown that E2 has a significant inhibitory effect on the growth of liver cancer cells and induces apoptosis in this cell line with a dose- and time-dependent manner. Similar results have obtained in other tissues by other investigators which reported that 17β-estradiol at a concentration 1 nM significantly increase apoptosis of MDA-MB-231 breast cancer cells(54). Besides, dietary phytoestrogens play a protective role against prostate and colon cancer and the formation of polyps(55,56). Furthermore, several experimental studies have reported that phytoestrogens reduce colorectal cancer development (57) and also similar study has shown that dietary phytoestrogens are associated with a reduction in colorectal cancer risk(58). Over the past decades, epidemiological studies have indicated that consumption of diets rich in phytoestrogens are associated with low risk of breast cancer(59). On the other hand, it has been demonstrated that phytoestrogen promotes cell death of human osteosarcoma cells U2OS by induction of apoptosis (60).

Many studies have reported a protective role of phytoestrogens against lung cancer and also reported that phytoestrogens reduce the risk of ovarian cancer and human cervical carcinoma HeLa cells through an apoptotic pathway(61). There are many researches which reported that phytoestrogens inhibit prostate cancer-cell growth during the promotional phase of the disease(62).

It has been reported that 17β-estradiol significantly inhibit the growth of ER-positive esophageal squamous carcinoma cell line (ES-25C) and the proliferation of the ER-positive KSE-1 esophageal squamous carcinoma cell line (63,64). Extensive researches have demonstrated that estrogen inhibits the proliferation, migration, and invasion of renal cell carcinoma and increases renal cell carcinoma apoptosis. All reports mentioned above confirm our finding but many studies have reported that phytoestrogen have stimulatory and proliferatory effects(65). Wu et al have demonstrated that steroid hormone has stimulatory effect on human gastric carcinoma cell line NUGC-3(26). On the other hand, it has been indicated that low concentrations of 17β-estradiol stimulate cell growth of MKN28, KATO III and MKN45 human gastric cancer cell lines (66) and also high phytoestrogen intake promote growth of estrogen-sensitive tumors.

Furthermore, it has been shown that 17β-estradiol administration exerts a growth-inhibitory effect on ER-positive cell lines (HGC-27, AGS) (67-69). In vitro study has demonstrated that 17β-estradiol increases both the invasion and proliferation of ovarian cancer cell.

It should be noted that phytoestrogens act through different mechanisms and pathways. They exert anticancer activity by two mechanisms including anti-estrogenic mechanism which is due to structural similarity with estradiol and anti-aromatase activity. They act through competing with endogenous estrogens in binding to estrogen receptors by which inactive enzyme involved in biosynthesis of estrogen from androgens such as aromatase (59,70). Many studies have reported that phytoestrogens exert antiproliferative effects by inhibition of tyrosine kinase activity, DNA-topoisomerase II and angiogenesis(71). Other studies have reported that estrogen act through four molecular pathways: ligand-independent, ligand-dependent, cell-surface (nongenomic) signaling and DNA binding-independent (72).

Phytoestrogens reduce cancer risk by binding to estrogen receptors (ER) or interacting with enzymes involved in sex steroid biosynthesis and metabolism (73). Other mechanism of phytoestrogens action reported by many studies include prevention of oxidation, induction of detoxification enzymes, antiproliferation, induction of cell cycle arrest and apoptosis (74-76). Many investigators have reported that phytoestrogens exert antitumorigenic activity on colorectal cancer by regulation of ER expression(77).

and also epidemiological and pathological data have reported that thyroid cancer is an estrogen-dependent disease and phytoestrogen consumption is associated with reduced risk of thyroid cancer(78).

In summary, based on data from many investigators, as mentioned above, 17-beta estradiol is a plant-derived estrogen-like compound with estrogenic activity and biphasic effects that acts through estrogen receptor probably by epigenetic pathways which need more researches. We did not perform enzyme activity assay related to methylation and histone modifications and also enzyme immunoassay related to protein levels but we will perform in next researches. It is important to note that, E2 had an apoptotic effect based on our data while other studies have reported that its effect is biphasic (inhibitory and stimulator effects).

CONCLUSION

Our findings suggest that, E2 may be a potent antiestrogenic compound and can effectively inhibit growth and induce apoptosis in PLC/PRF5 HCC cells. In future studies, the mechanisms and pathways of antiestrogenic effects of E2 on PLC/PRF5 should be evaluated.

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Fig. 2. Effect of E2 on PLC/PRF5 human hepatocellular carcinoma cell proliferation. Cells were seeded in 24 well plates and allowed to attach overnight and then were treated with E2 (1, 5, 10, 25, 50, 75, 100μM) for 24, 48 and 72 hours. Cell survival was determined by the MTT assay. Data are presented as mean ± standard error of the mean (SEM) from six independent experiments. Asterisks (*) indicate significant differences between treated cells and the control group. *P < 0.001 as compared to the control.

Fig. 3. Effect of E2 at a concentration of 25 μM on cell viability of PLC/PRF5 cells. The effect of E2 on the viability of PLC/PRF5 cells was determined by MTT assay at different timepoints (24, 48 and 72h). Mean values from the three experiments ± standard error of mean (SEM) are shown. Asterisks (*) indicate significant differences between treated cells and the control group. *P < 0.001.

Fig. 4. Effects of E2 on PLC/PRF5 cells apoptosis. The cells were treated with E2 (25 μM) for 24, 48 and 72 h and the apoptosis-inducing effect of E2 was investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidium iodide. Results were obtained from three independent experiments and were expressed as mean ± standard error of mean (SEM). *P < 0.001.