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Apoptotic Effect of Tamoxifen on Hepatocellular Carcinoma HepG 2 Cell Line

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ABSTRACT

Hepatocellular carcinoma (HCC) is the sixth most common cancer, and the third most common cause of cancer-related death worldwide. Major causes of hepatocellular carcinoma include hepatitis B, hepatitis C, alcoholic liver disease, and possibly nonalcoholic steatohepatitis. Tamoxifen (TAM) inhibits proliferation and induces apoptosis in many cancers such as breast cancer cell, gastric cancer BGC823 cell, SK-HEP-1 hepatoblasma cells and human cholangiocarcinoma cells. The aim of the present study was to analyze the apoptotic and antiprolifrative effect of TAM in the hepatocellular carcinoma HepG2 cell line. Materials and Methods: Cells were treated with various concentration of TAM and the MTT assay was used and then cells were treated with single dose of TAM (25μM) and flow cytometry assay was performed. Results: TAM inhibited the growth of HepG2 cells and induced apoptosis significantly with a time- and dose-dependent manner. Discussion: Our finding clearly indicated that TAM has a significant inhibitory effect and induces apoptosis with a dose- and time-dependent manner. Conclusion: TAM can significantly inhibit the growth of HepG2 cells and plays a significant role in apoptosis of this cell line.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer, and the third most common cause of cancer-related death worldwide1. This cancer accounts for between 85% and 90% of primary liver cancers. The geographic distribution of hepatocellular carcinoma is highly uneven. North America and Western Europe are generally considered to be low-incidence regions but in these regions the incidence of HCC is rising. In oncology cancer prevention may be categorized as primary or secondary. Primary prevention refers to the identification of genetic, biologic, and environmental factors. Secondary prevention refers to identification existing preneoplastic and early neoplastic lesions (2). Major causes of hepatocellular carcinoma include hepatitis B, hepatitis C, alcoholic liver disease, and possibly nonalcoholic steatohepatitis. Less common causes include hereditary hemochromatosis (antitrypsin deficiency) and autoimmune hepatitis (3).

Epidemiological studies, first in Africa (4) and Asia (5) and later in Europe (6), documented an important role of chronic infection with hepatitis B virus (HBV) in the etiology of hepatocellular carcinoma. Clinical case studies in Africa (7, 8) and in Europe (9) showed that chronic infection with hepatitis C virus (HCV) was also strongly linked to hepatocellular carcinoma. Both HBV and HCV have been declared to be carcinogenic to humans by the International Agency for Research on Cancer (IARC) (10). Meanwhile, evidence has emerged that tobacco smoking is strongly related to hepatocellular carcinoma (11).

Tamoxifen (TAM) is widely used in the treatment of many cancers such as breast cancer (12-14). It is used for patients with metastatic breast cancer and reduces the risk of recurrence and death from breast cancer when given as an adjuvant therapy (15-16). TAM induces cell-cycle arrest of breast cancer cells at low concentrations (0.1–1 μM), and induces apoptosis at pharmacological concentrations (above 5 μM) (17). It has been shown that TAM has proapoptotic effects in ER-negative breast cancer cells and other cell types such as those in malignant gliomas, pancreatic carcinomas, ovarian cancers, and melanomas (18-21). Recent studies have shown that TAM at pharmacological concentrations has an ionophoric effect on cell membranes permeability and intracellular pH, which leads to decreased viability and death of the cells (18). Other studies have reported changes in membrane fluidity and

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alterations in intracellular calcium fluxes (22). TAM induces apoptosis and inhibits growth of gastric cancer BGC823 cell with a concentration dependent manner (23).
Tamoxifen can induce apoptosis of SK-HEP-1 hepatoblasma cells (24, 25). It is an important therapeutic agent for patients with esterogen receptor positive breast cancers (26). It has been noted that tamoxifen inhibits the growth of human cholangiocarcinoma cells in culture and inhibits tumor growth in the mice (27).
Since there is few studies for tamoxifen effect on HepG2 cell line and we cannot find any data about effect of tamoxifen on HepG2 cell line (in vitro),therefore ,this study was designed to investigative the apoptotic and anti-proliferative effects of tamoxifen 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. Tamoxifen, 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 (HepG2 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 tamoxifen (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 tamoxifen at different times using inverted microscope (Nikon, TE 2000-U, Japan).

DETERMINATION OF IC50 VALUE BY MTT ASSAY

The effect of tamoxifen 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 tamoxifen 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 tamoxifen 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 tamoxifen, the cells were seeded in triplicate in 24-well plates and treated with tamoxifen 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 tamoxifen (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 tamoxifen at concentration of 25 μM in different time periods were analyzed by using the MTT assay. The amounts of reduced MTT in the all groups treated with tamoxifen were significantly lower than that of the control group ($P < 0.001$). The percentage of living cells in treatment groups at different time periods (24, 48 and 72h) were 53, 51 and 49%, respectively at a concentration of 25 μM of tamoxifen. This experiment was repeated three times for each group (fig.1).

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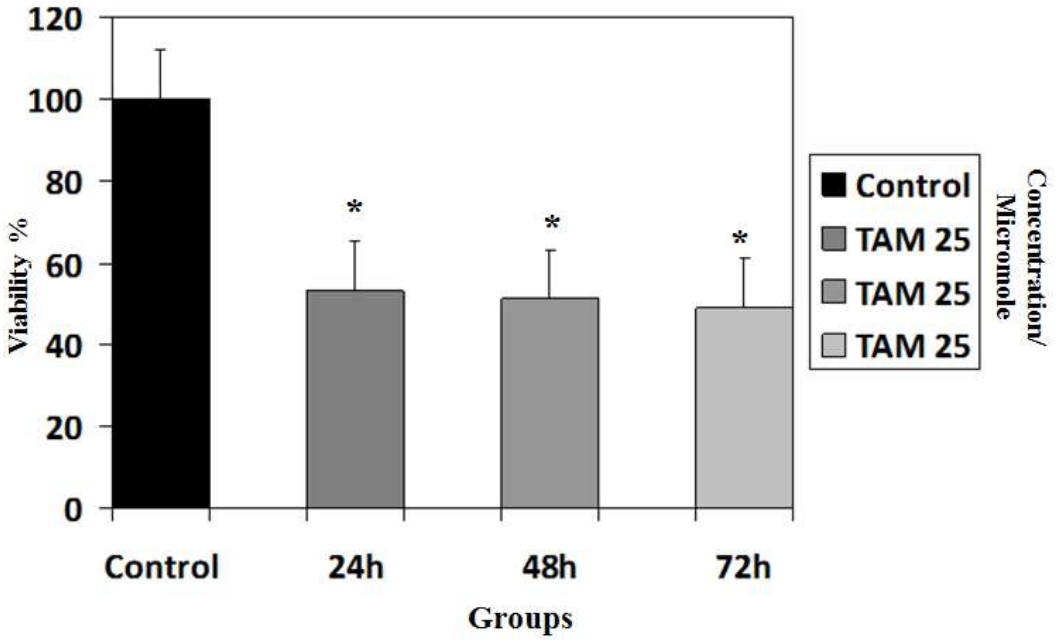

Fig 1. Effect of TAM on cell viability of HepG2 cells. The effect of TAM on the viability of HepG2 cells was determined by MTT assay at different time periods (24, 48 and 72h). Mean values from the three experiments± standard error (S.E.M) are shown. Asterisks indicate significant differences between 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 tamoxifen 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 tamoxifen 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.2 and 3). 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.

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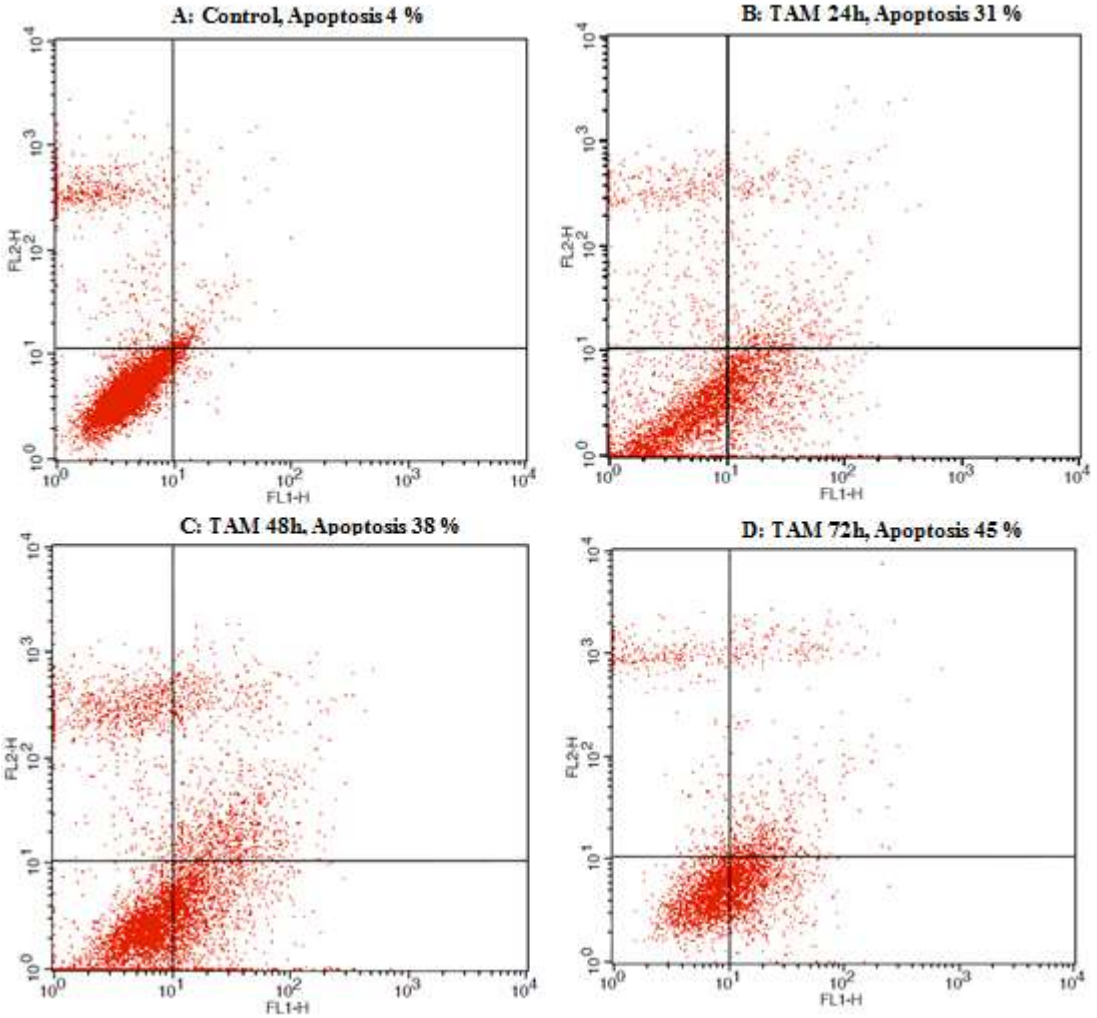

Fig 2. Effect of TAM on HepG2 cells apoptosis. The cells were treated with TAM (25 μM) for 24, 48 and 72h and the apoptosis- inducing effect of TAM was investigated by flow cytometric analysis of HepG2 cells stained with Annexin V and propidium iodids.

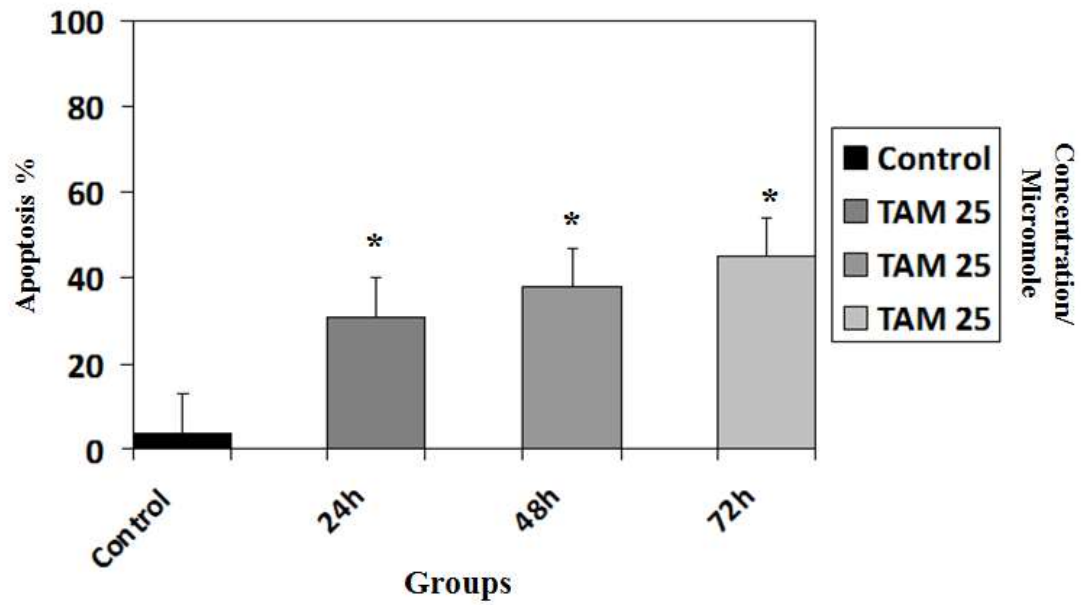

Fig 3. Apoptotic-inducing effects of TAM (25μM) for 24, 48 and 72h. Result were obtained from three independent experiments and were expressed as mean± standard error of mean (S.E.M). Asterisks indicate significant differences between treated cells and the control group. P< 0.001, n=3, A: control, B: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

DISCUSSION

Hepatocellular carcinoma is the dominant form of primary liver cancer (28). Approximately 70%–90% of patients with hepatocellular carcinoma have a background of chronic liver disease and cirrhosis, with major risk factors for developing cirrhosis including chronic infection with hepatitis C virus (HCV), hepatitis B virus (HBV), and alcoholic liver disease(29,30). Other risk factors for developing hepatocellular carcinoma include intake of aflatoxin-contaminated food, obesity, diabetes, certain hereditary conditions such as hemochromatosis, and some metabolic disorders (31, 32).

Tamoxifen can induce apoptosis of SK-HEP-1 hepatoblasma cells (24, 25). It is an important therapeutic agent for patients with ER positive breast cancers (26). It has been demonstrated that tamoxifen inhibits the growth of human cholangiocarcinomas cells in culture and inhibits tumor growth in the mice (27).

In the present study, tamoxifen inhibited growth of liver cancer cells and induced apoptosis in HepG2 cell line with a time-dependent manner. This is consistent with other reports that have shown that tamoxifen (1, 10, or 20 μM up to 72h) inhibits cell viability in HepG2 cells depends on drug concentration. It may be mediated by a down-regulation of telomerase activity (33). It has been noted that tamoxifen (20 μM for up to 72 h) can decrease cell proliferation and induce apoptosis in HepG2 cell line, which is due to cytostatic and cytocide effects, the latter may be mediated by a down-regulation of survivin expression (34) and also tamoxifen (5–10 μM) causes dose-dependent *in vitro* growth inhibition of two human cholangiocarcinoma cell lines (OZ and SK-ChA-1) (35).

Opposite of our data, studies with tamoxifen indicate that tamoxifen with daily doses of 40 mg or 30 mg for more than 2 years increases the risk of endometrial cancer (36). Other studies have shown that tamoxifen treatment there has a significant increase in the incidence (26%) of uterine adenocarcinomas and a 9% incidence of squamous cell carcinomas of the vagina/cervix (37). Finally it has been reported that tamoxifen at low concentrations (10^{-9} and 10^{-11} m) causes stimulation of cell proliferation in a cervical cancer cell line **(38).** Considering the results of our research, TAM maybe good candidate for HCC treatment.

CONCLUSION

The results of the present study indicate that TAM can inhibit proliferation and induce apoptosis in human HepG2 cell line. This work provides new insights into the molecular mechanisms of TAM in these cells.

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