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The effects of hesperetin on apoptosis induction and inhibition of cell proliferation in the prostate cancer PC3 cells

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ABSTRACT

Introduction: Prostate cancer is the second leading cause of cancer-related deaths and the most common cancer diagnosed in men in the United States and Europe. Hesperetin, a member of the flavonoids with antioxidant property, is found in fruits such as oranges and red fruits. This study was undertaken to evaluate the effects of hesperetin on apoptosis induction and inhibition of cell proliferation in the prostate cancer PC3 cells.

Methods: PC3 cell line was cultured in standard condition. The cells were exposed to different concentrations of hesperetin (0-1000 µM) for 48 hours. Cell viability was measured by MTT assay. Apoptosis induction was assessed by Annexin V-FITC by flow cytometry analysis.

Results: The PC3 cells exposed to hesperetin (0-1000 µM) exhibited an IC_{50} (inhibitory concentration of 50%) about 450 µM. At different concentrations of hesperetin (400, 450 and 500 µm), the apoptosis increased slightly (not significant) in treated PC3 cells compared to the control group (5.4%, 7.8% and 9.1% respectively vs. 4.2%).

Conclusion: These results clearly show that hesperetin can lead to inhibition of PC3 cells proliferation.

Implication for health policy/practice/research/medical education:
Hesperetin has anti-cancer properties against different cell lines and this effect seems to be through the inhibition of cell proliferation with the induction of cell cycle arrest at the G1 phase. These results can be a step toward targeted combination or alternative chemotherapy in the prostate cancer.

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Introduction

Prostate cancer is the second major cause of cancer-related deaths and the most common cancer diagnosed in men in the United States and Europe. Prostate cancer incidence increases with age (1,2). It is initially androgen-dependent (AD) but it will progress to become a more invasive and androgen-independent form with resistance to treatment (3). The apoptotic process is an important target in treatment of cancer. Apoptosis or programmed cell death is characterized by process of autonomous cellular elimination which avoids eliciting inflammation (4).

Apoptosis can be triggered by 2 main pathways; one is the extrinsic death-receptor pathway and the other is the intrinsic mitochondrial pathway (5). The main treatment for most cancers is chemotherapy but it has side effects such as decreasing the number of peripheral blood cells or toxic adverse effects (6). Thus, in recent years the medicinal plant compounds are used to treat cancer. Flavonoids and flavonoid-rich food sources have been reported to be used for the treatment of several diseases with their powerful antioxidant activities (7).

Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone), a member of the flavonoids, is found in fruits such as oranges and red fruits (8). Many reports have shown that flavonoids have various anticancer effects such as kinase activity, the inhibition of cell proliferation, and the induction of apoptosis on cancer cells (9). Hesperetin and its metabolites have several biological activities such as an-

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tioxidant, anti-inflammatory, and lipid lowering effects (10). Hesperetin has also been shown to act as a neuroprotective compound (11) and some studies are indicative of hesperetin neuroprotective properties against apoptosis (12,13). Therefore, considering the antioxidant properties of hesperetin, the aim of this study was to examine the effects of hesperetin on apoptosis induction and inhibition of cell proliferation in the prostate cancer PC3 cells.

Materials and Methods

Reagents

The human PC3 prostate cancer cells were purchased from Pasteur Institute of Iran (Tehran, Iran). RPMI1640 medium, PEN/STREP, dimethyl sulfoxid (DMSO), trypan blue, and hesperetin were obtained from Sigma (St. Louis, MO). Fetus bovine serum (FBS) was purchased from GIBCO (USA) institute. MTT were acquired from Merck (Darmstadt, Germany).

Cell culture and cell count

The human PC-3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin (PS). The cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Total and viable cell counts (survival rate) were determined by standard hemocytometer procedures with the trypan blue exclusion test. Live viable cells were seen as colorless (impermeable to the dye due to intact membrane) and dead cells were seen as blue (permeable to dye due to disruption of cell membrane).

Determination of cell viability (MTT assay)

The cytotoxic effects of the hesperetin on the PC3 cells were determined by the MTT assay (14). The principle behind this technique depends on the capacity of living cells to reduce tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a formazan crystal in their metabolizing mitochondria. Briefly, PC3 cells were seeded at an amount of 5×10^3 cells per well in 96-well plates and allowed to grow overnight. The cells were treated with different concentrations (0-1000 µM) of hesperetin (solution in DMSO with 0.1% final concentration). After incubation for 48 hours, supernatant was discarded and was added 100 µL colorless RPMI. Then, adding 10 µL MTT (5 mg/mL in phosphate buffered saline) to each well and incubated for 4 hours. After the removal of the medium, 150 µL DMSO was added to each well and shaken gently and carefully. The absorbance was read at a wavelength of 490-620 nm using an ELISA microplate reader (State Fax® 2100, Awareness, USA).

Analysis of apoptosis and necrosis

The analysis of apoptosis was conducted by flow cytometry. Apoptosis was measured by kits specifically (BD Biosciences) according to the manufacturer's instructions. Briefly, PC3 cells were cultured in 6-well plates and allowed to attach to plate overnight. The cells were treated with 450 µM of hesperetin for 48 hours and they were harvested by trypsinization and, after washing with cold PBS, were diluted with 500 µL of binding buffer, and incubated in 10 µL of Annexin-binding buffer with 2 µL of Annexin V-FITC for 20 minutes in the dark. Then, the cells were acquired and analyzed by flow cytometry (15).

Statistical analysis

The data were expressed as mean ± S.D. They were analyzed by SPSS software (version 20) and Graph Pad Prism (version 5.01, USA). Nonparametric data were analyzed with Kruskal-Wallis test. A $P \leq 0.05$ was considered significant. All experiments were repeated at least 3 times.

Results

Figure 1 shows that hesperetin has decreased significantly cell proliferation in a dose-dependent manner ($P < 0.05$) after 48 hours. The cells that exposed to hesperetin (0-1000 µM) exhibited an IC_{50} (inhibitory concentration of 50%) about 450 µM. In this concentration, hesperetin eliminated 50% of the cells.

Effect of hesperetin on apoptosis in PC3 cells

Figures 2 and 3 show flow cytometric analysis using Annexin V staining for apoptosis in PC3 cells. Apoptosis in PC3 cells was slightly induced (not significantly) 5.4%, 7.8% and 9.1% at 400, 450, and 500 µM of hesperetin, respectively.

Discussion

Chemotherapy is considered as the main treatment for most types of cancers but because of side effects, major efforts have been made to recognize natural compound that can prevent the development of cancers. Many natural compounds have been found to induce apoptosis in various cancer cells and there are obvious evidences indicating that these compounds are potent inhibitors of cancer cell

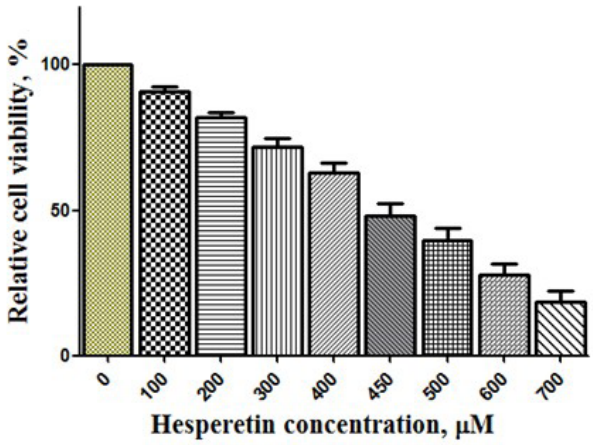

Figure 1. Inhibition of cell proliferation by hesperetin for 48 h. The cells were cultured at the density of 5×10^3 cells per well in 96-well plates and were allowed to grow overnight. The cells were treated with different concentrations (0-1000 µM) of hesperetin (solution in DMSO 0.1%) after incubation for 48 hours.

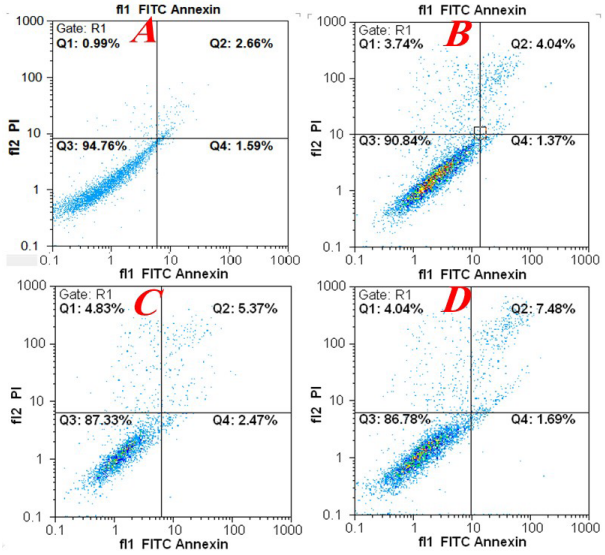

Figure 2. Effect of hesperetin on apoptosis in PC3 cells. The cells were cultured for 24 hours. Then, the cells were treated with different concentrations of hesperetin (0, 400, 450, 500 µM) for 48 hours. The appearance of apoptosis cells was detected by flow cytometry using Annexin V-FITC staining. A; Control without treatment, B, C and D the cells were treated with 400, 450, and 500 µM of hesperetin, respectively.

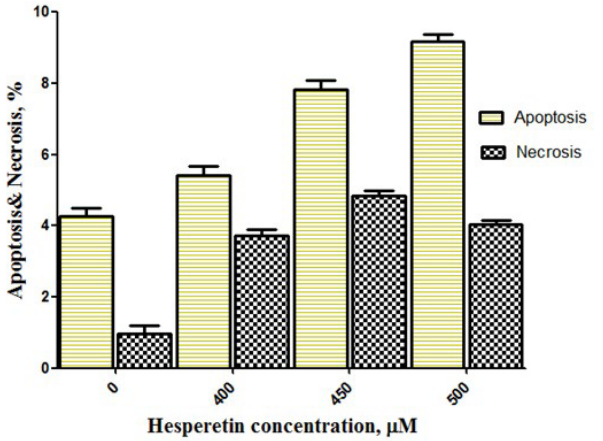

Figure 3. The effect of different concentrations of hesperetin on PC3 cells apoptosis after 48 hours incubation compared to control group. No significant change ($P > 0.05$) was observed in apoptosis and necrosis between hesperetin-treated PC3 cells and control group cells different concentrations of hesperetin.

proliferation (16,17). This beneficial effect has often been attributed to the high content of naturally-occurring antioxidants, such as vitamins and flavonoids. In particular, studies have indicated a specific link between citrus intake and the reduced risk of diseases such as cancer (18,19).

In the present study, we analyzed the effects of hesperetin (a flavonoid) on PC3 cells. The effects of this antioxidant were assessed on growth and proliferation of PC3 cells in vitro using an MTT assay or cytotoxicity assay test (Figure 1). Hesperetin in 450 µM led to the reduction of cell viability as reported by other investigators (20,21). Our results of flow cytometry indicate that apoptosis increased slightly (not significantly) in hesperetin-treated PC3 cells compared to the control group (Figures 2 and 3). Previous studies have also shown that hesperetin treatment leads to the inhibition of cell proliferation with the induction of cell cycle arrest at the G1 phase (22). Nevertheless, Palit et al (23) showed that hesperetin would induce apoptosis in breast carcinoma by triggering aggregation of reactive oxygen species (ROS) and activation of ASK1/JNK signaling pathway (23) which runs counter to our findings. On the other hand, some studies have demonstrated that hesperetin has antioxidant, anti-proliferation, and apoptosis effects (24). Also, it was found that hesperetin at 100 nM was effective in preventing neuronal apoptosis via a mechanism involving the activation/phosphorylation of both ERK1/2 and Akt/PKB signaling pathways (11). Choi et al showed that hesperetin and epigallocatechin gallate exerted antiapoptosis effects in endothelial cells exposed to oxidized LDL. They showed that hesperetin was mediated by interrupting both JNK and p38 MAPK-responsive death pathways (25). Also, in another study it was found that protective effects of hesperetin and naringenin against apoptosis in ischemia/reperfusion induced retinal injury in rats. As a result hesperetin and naringenin can prevent harmful effects induced by I/R injury in the rat retina by inhibiting apoptosis of retinal cells, which proposes that those flavanones have a therapeutic potential for the protection of ocular ischemic diseases (10). Therefore, in this study the prevention of PC3 apoptosis probably resulted in the changes of cellular signaling proteins.

In this study, we did not evaluate the effect of hesperetin on cellular signaling proteins such as the nuclear translocation level of p65, ERK1/2, Akt/PKB, and regulatory effectors such as Bcl2 levels, and Bax levels. These factors can influence cell apoptosis and survival. Therefore, we suggest that future studies focus on the effects of EA on other cell signaling and effector proteins.

In conclusion, our data confirmed that hesperetin led to the reduction of PC3 cells without considerable increasing apoptosis considerably. Therefore, the study of the mechanism of apoptosis induction could be a step of progress toward target therapy which might be considered in the future studies.

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Authors' contributions

MS performed the experimental work and helped the writing, EH contributed in the conception of the work, design, writing, analysis, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work, PB led the flowcytometry analysis.

Conflict of interests

The authors report no conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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