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Effect of crocin and doxorubicin / radiation on the breast cancer cell line, Michigan Cancer Foundation-7

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ABSTRACT

Crocin is the main carotenoid of saffron showing anticancer properties. Doxorubicin as a chemotherapy drug and X-ray or gamma radiation therapy are used extensively in the treatment of breast cancer. However their side effects limited their use. The aim of this study was to investigate the apoptosis of Michigan center foundation-7 breast cancer cells in monolayer culture (in vitro), using crocin, doxorubicin, radiation, crocin-radiation, and crocin-doxorubicin.To explore the effect of crocin, doxorubicin and radiation, Michigan center foundation -7 cell line was cultured and treated with different concentrations of crocin and doxorubicin. MTT assay was used to evaluate the toxicity, PI flowcytometry was used to evaluate the apoptosis and Western blotting was applied to investigate the protein expression of p53, PARP, and caspase-7.According to the MTT assay, crocin can decrease growth of Michigan center foundation-7cell in a dose and time dependent manner. The results of flowcytometry also showed that apoptosis rate was significantly higher in the combined test than Separate tests. Western blot analysis also revealed that the proteins expression in combined groups was much than separated groups. This study revealed the expression of apoptotic proteins in the combined therapy of saffron and radiation or saffron and drug was significantly higher than that in using radiation or drug alone.

KEY WORDS: CROCIN, DOXORUBICIN, GAMMA RAY, APOPTOSIS, BREAST CANCER

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Introduction

Cancer is the second leading cause of death in the world. In recent years its rate has grown even more than twice. Breast cancer is the most common cancer (23% of all cancers) with the highest mortality (16%) among all women's' malignancies. Although the incidence of cancer is low in Asia, but there is more abundance of death due to cancer in most of the Asian countries than that in Western countries. In Iran breast cancer has the most incidences among all malignancies in women. In previous studies it has been found that the incidence of breast cancer in Iran is lower than that in developed countries. However, it is still considered as the most common cancer in Iranian women and there have increased in incidence in two past decades (Harirchi, Kolahdoozan et al. 2011).

According to the report of World Health Organization, breast cancer is increasing in developing countries based on some factors such as increase of life expectancy, urbanization and following up the western life style. In spite of using three important methods for breast cancer treatment including surgery, chemotherapy and radiotherapy, it has high mortality rate indicating the inefficiency of these therapeutic methods (Møller, Wallin et al. 1996).Saffron is commonly consumed in different parts of the world as a medicinal drug to treat several health disorders(Schmidt, Betti et al. 2007).

Crocin is the main carotenoid of saffron showing antitumor properties (Jaliani, Riazi et al. 2013). Currently, it has been proved clinically that crocin may improve the anti-tumor effect as well as reduce the doses of chemotherapeutic agents(Naghizadeh, Boroushaki et al. 2008). Noticeably, crocin inhibits the growth of cancer cells significantly with no side effects on normal cells (Sun, Xu et al. 2013 and Tazhibi and Feizi 2014) .

Doxorubicin is an Anthracycline drug, which is traditionally used for breast cancer treatment. Anthracyclines induce double-stranded DNA break associated with the production of free radicals. The free radicals could lead to inhibition of mitochondrial respiratory chain enzymes and oxidation of membrane lipids. At the cellular level, doxorubicin perches between two nitrogen bases of double stranded DNA helix, causing inhibition in DNA polymerase and DNA-dependent RNA polymerase function. This function can lead to suppression of DNA and RNA synthesis anddamages DNA repair mechanism. Doxorubicin also changes Topoisomerase II activity. Doxorubicin has a stronger influence on the S phase of the cell. However some problems such as hematopoietic suppression, nausea, vomiting, extravasation, alopecia and cardiotoxicity lead doxorubicin's success to become incomplete (Czeczuga-Semeniuk, Wołczynski et al. 2004, Howe Luzhna and Kovalchuk 2010 and Octavia, Tocchetti et al. 2012).

Ionizing radiation has ability to change the organic bases of the nucleotides, the sugar part of DNA molecule and most importantly cut the DNA molecule(Scott and Pandita 2006). Studies show that even a small number of double stranded DNA breaks after the exposure of ionizing radiation could be associated with cell death.DNA is can damage from ionizing radiation such as Radiotherapy because it includes information to encode biomolecules then single strand break (SSB), double strand break(DSB), and dimerization could threat the cell viability strongly. Therefore, the ionizing radiations have potential to induce DNA damage and apoptosis.(Criswell, Leskov et al. 2003 Sun, Zhao, 2013)

Apoptosis is a type of common cell death in eukaryotes.This process is performed during embryonic stage and tumor suppression (Nicoletti, Migliorati et al. 1991). The aim of this study was to investigate the apoptosis of breast cancer cells (Michigan center foundation-7 cell line) in monolayer culture (in vitro), using crocin, doxorubicin, radiation, crocin- radiation and crocin-doxorubicin.

MATERIAL AND METHODS

CELL LINES AND REAGENTS

The human breast cancer cell lines MICHIGAN CANCER FOUNDATION-7, a non-invasive estrogen receptor (ER) positive, were obtained from Cell Bank of the Pasteur Institute (Tehran, IRAN). Trypsin and crocin were purchased from Sigma. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) and Propidium iodide (PI), purchased from Sigma. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) high glucose with 5% fetal bovine serum (Gibco). Doxorubicin was prepared from Sobhan Darou (Tehran, Iran). Rabbit monoclonal antibody of caspase-7, caspase-9, PARP and mouse monoclonal antibody of p53 were bought from Cell Signaling (USA).

CELL CULTURE AND RESEARCH METHODS

Michigan center foundation-7 cells were cultured in the DMEM high glucose medium, supplemented with 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere at 370C and 5% CO2.Cellsin the logarithmic growth period were selected for experimental studies.

MEASUREMENT OF THE SURVIVAL RATES OF MICHIGAN CENTER FOUNDATION-7 CELLS WITH MTT METHOD

25000 cells were seeded in the 24-well plate with 1ml of culture medium for 24 h. Different concentrations of 1.5mg/ml, 2.5mg/ml, 3.5mg/ml, 4.5mg/ml and 6mg/ml for the crocin and 0.01µM/ml, 0.03 µM/ml,0.05µM/ml, 0.1µM/ml, 0.5µM/ml and 1µM/ml for the doxorubicin were examined separately to get IC50 (Inhibition concentration) for Michigan center foundation-7 at the minimum possible time. On this base the incubation times for crocin were used 24 h, 48 h and 72 h and for doxorubicin 48 h. Then the medium was being removed and cells were incubated with 100 µl (5mg / ml dissolved in PBS) MTT and 900 µl medium culture at 370C for 4 h. For each well the supernatant was discarded, 500ul DMSO was added and the mixture was suspended. The light absorbance (A) was measured at 570 nm wavelength using ELISA. Finally survival rate calculated as follows: survival rate of tumor cells (%) = experimental group A value/control group A value×100%.

All above steps were done for crocin and doxorubicin separately. As a result concentrations of 2.5mg/ml for crocin and 0.01µM/ml for doxorubicin were selected as the optimized combination. Then four groups of Michigan center foundation-7 were selected to study three methods of treatments as follows: a control group, second group irradiated by 2Gy Gamma radiation (with cobalt source), and the third group received 2.5mg/ml crocin with 0.01µM/ml doxorubicin and finally fourth group was studied by 2.5mg/ml crocin with 2Gy Gamma radiation. The experiment times were 24 h, 48 h and 72 h for crocin groups and 48 h for another group, after the treatment with drug. All trials were repeated three times.

DETERMINATION OF CELL APOPTOSIS BY FLOWCYTOMETRY

Apoptotic cells were revealed by the flowcytometry using PI staining to detect the so-called sub-G1 peak (Riccardi and Nicoletti 2006).This process leads to measure DNA content after staining nucleic acid with specific fluorochromes(Riccardi and Nicoletti 2006) For this assay MICHIGAN CANCER FOUNDATION-7 cells were cultured in a 6-well plate (70000 cells per well) and treated with 2.5mg/ml crocin for 24 h, 48 h and 72 h. The second group of cells was treated with .01µM/ml of doxorubicin and the third group was irradiated with 2 Gy gamma. To evaluate the combined therapy, the fourth group was treated by 2.5mg/ml crocin and 0.01 µM/ml doxorubicin for 48 h and the fifth group was treated by 2 Gy gamma and 2.5mg/ml crocin for 24 incubation. Then for all groups beside the control group the flow cytometric analysis was being done.

WESTERN BLOT TO DETECT THE EXPRESSION OF CASPASE-7 AND CASPASE-9 AND P53 AND PARP OF MICHIGAN CENTER FOUNDATION-7 CELLS

Michigan center foundation-7 cell lines were classified and treated similar to two pervious methods. Then treated cells were detached by trypsinization, washed with PBS, centrifuged 4000 rpm for 5 minutes added with RIPA (lysis buffer). In next step all samples were put in ice for 30 minutes and vortexed every 5 minutes until those would have been homogenized well. Then samples were centrifuged 13000 rpm for 20 minutes at 4^{0}C. The supernatant was taken out and the concentration of protein was being measured by Bradford method. Protein samples were divided into smaller amounts and kept at -80^{0}C.

To do western blot at first running buffer and transfer buffer were prepared and three steps were followed as: a) proteins were separated according to molecular weight by gel electrophoresis, b) transferred to nitrocellulose membrane, c) then the desired protein was specified with the primary antibody and shown with the secondary antibody. Finally antibody bonds appeared with ECL on the film. The thickness of each bond was directly related to the amount of protein.

RESULTS

CHANGES IN SURVIVAL RATES OF MICHIGAN CENTER FOUNDATION-7 CELLS

This study revealed the cell growth of MICHIGAN CANCER FOUNDATION-7 cell line was inhibited by crocin in a dose and time dependent manner (Figure 1 A-C). There were significant differences among different groups (P value <0.05). IC50 was shown for 3.5 mg/ml crocin in 48 h by MTT (Figure 1 B).The resultes of Crocin MTT asseys are similar to results of Vali and changizi 2015.

Different concentrations of doxorubicin for 48 incubation could suppress MICHIGAN CANCER FOUNDATION-7 cell line in a dose dependent manner (P value <0.05) (Fig.1D). For this drug IC50 was obtained 0.1 µM/ml in 48 h by MTT assay. This research showed combination of different concentrations of crocin and 0.01 µM/ml doxorubicin, may have stronger inhibition effect than the single agent on breast cancer cells. Similarly it was shown with combination of 2.5mg/ml of crocin and

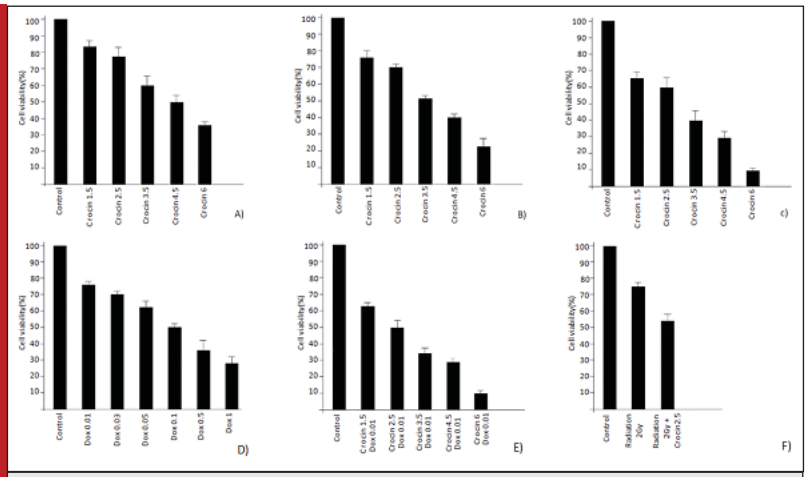

FIGURE 1: The inhibition effect of crocin, Doxorubicin, Radiation and combination of them on MICHIGAN CANCER FOUNDATION-7 cells, measured by MTT (A) crocin 24h, (B) crocin 48h, (C) crocin 72 h. (D) Doxorubicin 48h (E) combination of crocin in all concentration 48h with 0.01uM/ml Doxorubicin 48h (F) combination of crocin 48h with radiation 24h.

2Gy radiation the survival rate was significantly lower (P value <0.05) (Fig. 1E _

APOPTOTIC CHANGES OF MICHIGAN CANCER FOUNDATION-7 CELLS

Apoptosis following treatment with crocin, doxorubicin, radiation and combination of them was measured by flowcytometry using PI staining to detect the sub-G1 peak resulting from DNA fragmentation. The results showed apoptosis rate with crocin to be increased with duration of time (P < 0.05) (Fig. 2 A _ D). Also this study revealed the combined treatment of crocin and doxorubicin or crocin and radiation have more apoptotic effect than the single agent (P < 0.05) (Fig. 2 E _ H).

EXPRESSION OF CASPASE-9, CASPASE-7, P53 AND PARP IN MICHIGAN CANCER FOUNDATION-7 CELLS

With exposure of 2.5 mg/ml and 4.5 mg/ml crocin to MICHIGAN CANCER FOUNDATION-7 cells for 48h,

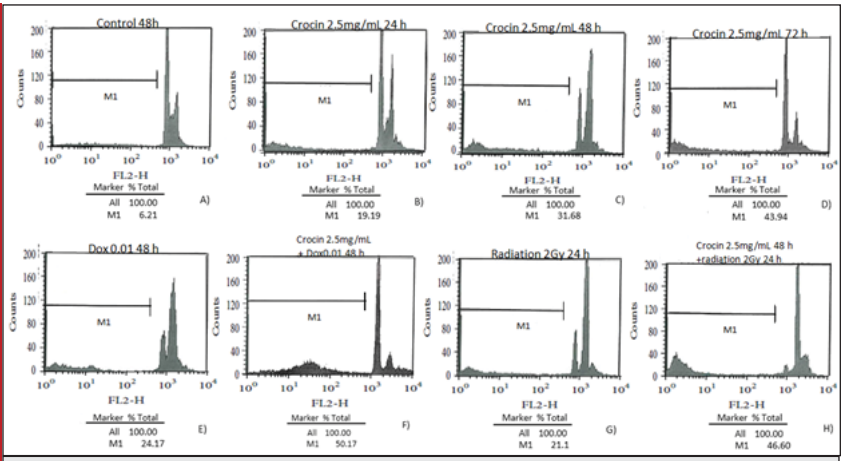

FIGURE 2: Flowcytometry histograms of PI-stained MICHIGAN CANCER FOUNDATION-7 cells and Sub-G1 peak means apoptotic cells.(A) control 48h (B) Crocin 2.5 mg/ml 24h (C) Crocin 2.5 mg/ml 48h (D) crocin 2.5 mg/ml 72h (E) doxorubicin .01uM/ml 48h (F) crocin 2.5mg/ml 48h + doxorubicin .01uM/ml (G) radiation 2Gy (H) crocin 2.5 mg/ml 48h + radiation 2Gy 24h.

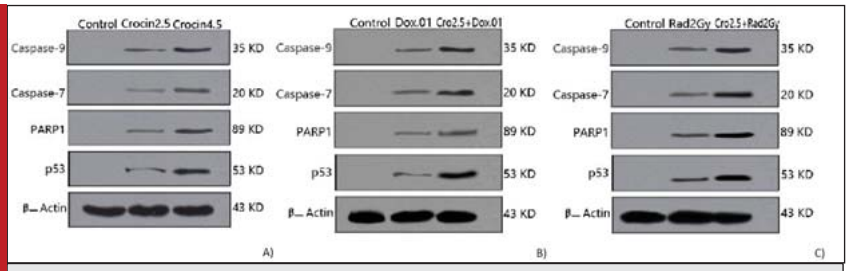

FIGURE 3: Evaluating the expression of caspase-9, caspase-7, p53 and PARP (A) in a concentration of 2.5 and 4.5 mg/ml in 48 h. (B) in dox .01uM/ml , crocin 2.5mg/ml + dox.01uM/ml (c) in radiation 2Gy , crocin2.5+radiation2G.

expression of p53, PARP, caspase-9 and caspase-7 was detected. The results showed that the expression of all four proteins was in a dose dependent manner (Fig. 3 A). Also expression of these proteins was evaluated in combination of crocin with doxorubicin or radiation. The results showed that the expression of apoptotic proteins in the combined treatments were significantly higher than the single groups (Fig. 3 B_C).

DISCUSSION

Cancer is the second leading cause of death in the world. In recent years its rate has been grown even more than twice. Breast cancer is the most common cancer (23% of all cancers) with the highest mortality (16%) among all malignancies in women. Therefore breast cancer remains one of the main health problem in the word (Harirchi, Kolahdoozan et al. 2011). Studies show that increased incidence of the breast cancer is higher in developing countries and life expectancy of patients is lower (Shibuya, Mathers et al. 2002). The reasons of cancer are associated with the environmental factors such as air pollution, stress, diet and life style of the people. It has been found that the consumption of foods that have antioxidant properties, is effective in reducing the risk of cancer (Ren, Qiao et al. 2003).

On the other hand, despite the use of therapeutic options including surgery chemotherapy and radiotherapy for patients mortality remains high. It indicates the inefficiency of these ways. Moreover destructive effects of chemotherapy and radiation on normal cell division are also other disadvantages associated with the therapeutic process (Chabner Ba Fau - Friedman and Friedman). For example, while doxorubicin is a valuable anticancer agent, cardiotoxicity and drug resistance are two main problems of this drug (Kaye and Merry 1985, Weiss 1992).

Crocin is the main carotenoid of saffron with antitumor properties (Jaliani, Riazi et al. 2013). Study on HepG2 cell line showed telomerase activity in the nucleus would decrease with increasing concentration of crocin and it would lead to inhibition of proliferation and apoptosis (Noureini and Wink 2012). Our study could approve this result. It was also found that DNA fragmentation and cell cycle arrest are the main signs of apoptosis in pancreatic cancer cells treated with crocin(Bakshi, Sam et al. 2010). Gupta and colleagues in October 2014 investigated the synergistic effect of crocin and cisplatin on MDA MB-231 cells and MCF_7. It was found that lower concentrations of cisplatin along with crocin can achieve the desired result (Gupta, Jhamb et al. 2014). Notably, crocin significantly inhibits the growth of cancer cells with no effects on normal cells (Sun, Xu et al. 2013).

Consistent with the results of other studies, the results of this study also confirms the effectiveness of crocin against breast cancer Michigan center foundation-7 cell line. To evaluate the survival rate of Michigan center foundation-7 cells in treatment with doxorubicin, crocin, radiation and combined therapy MTT assay was used. This study showed treatment of Michigan center foundation -7 cells with crocin was time and dose-dependent to decrease the rate of proliferation. For example when the concentration of crocin increased from 1.5 mg/ml to 6mg/ml in 48h, the survival rate of MCF-7 cells reduced from 75% to 23%. Also the survival rate of MCF-7cells reduced from 75% to 22% in treatment of cells with doxorubicin from .01 µM/ml to 1µM/ml in 48h. It means doxorubicin reduces the survival rate in a dose dependent manner.

Cellular stresses such as ionizing radiation, UV and chemical carcinogens can activate p53, including damage to DNA, oncogene expression, hypoxia and nucleotide depletion (Giaccia and Kastan 1998). According to the type and severity of toxicity, p53 protein causes cell cycle arrest or cell death through apoptosis that the former cause to repair DNA and the second led to remove from cell replication(Bouvard, Zaitchouk et al. 2000) . P53 causes to release cytochrome C from mitochondria and induces the expression of Apaf-1 forming the apopto-

some (Kannan, Kaminski et al. 2001). Apoptosome leads to activate caspase-9 following with executive caspases such as 3,6 and 7 (Hengartner 2000). The caspase-activated DNase cut DNA between nucleosomes (Pecorino). This process leads to measure DNA content after staining nucleic acid with specific fluorochromes(Riccardi and Nicoletti 2006) . PI is a fluorogenic compound that binds to nucleic acids, so that fluorescence emission is proportional to the DNA content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they show a wide hypodiploid (sub-G1) peak, that easily discriminates from narrow peak of normal cells (diploid cells) (Riccardi and Nicoletti 2006).

Michigan center foundation is a cancer cell line with defect in caspase-3 and is relatively insensitive to many chemotherapy drugs (Yang, Sladek et al. 2001). PARP as the cellular protein is cleaved specifically in apoptosis. Particular proteolysis of PARP happens in the DNA binding domain. Caspase-3 and caspase-7 are the most effective proteases for PARP cleavage (Herceg and Wang 2001). Therefore PI flowcytometry and western blot were used to evaluate breast cancer cells apoptosis in our study. Flowcytometry showed to treat MCF 7cells for 48 hours with 0.01 µmol/ml doxorubicin causes 24.17% apoptosis. The combined therapy of 2.5 mg/ml crocin and 0.01µm/ml Doxorubicin for 48 hours with a synergistic effect caused 50.17% apoptosis. Also the combined therapy of crocin and gamma radiation with a synergistic effect could cause 46.60% apoptosis in breast cancer cells.

CONCLUSION

This study revealed the expression of apoptotic proteins in the combined therapy of saffron and radiation or saffron and drug was significantly higher than that in using radiation or drug alone. Finally it was found that crocin could be an appropriate supplement for treatment of breast cancer by reducing the dosage and harmful effects of drugs or radiation.

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CONFLICT OF INTEREST

There is no conflict of interest

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