

The Synergic Effects of *Crocus Sativus L.* and Low Frequency Electromagnetic Field on VEGFR₂ Gene Expression in Human Breast Cancer Cells

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Abstract

Background: Angiogenesis, which is required for embryonic development and many physiological events, plays crucial role in many pathological conditions such as tumor growth and metastasis. Recent studies indicate anticancer and antitumor properties of saffron against human cancers. Many processes are affected by Electromagnetic Field (EMF) and its effect on proliferation and gene expression were examined. In this experimental study, the synergic effects of saffron and EMF on VEGFR₂ gene expression in MCF7 cells were investigated.

Methods: Saffron was extracted using freeze dryer. MCF7 cells were grown in RPMI 1640 medium supplemented with 10% FBS and incubated at 37 °C with 5% CO₂. After 24 hr cells were treated with saffron extract at concentrations of 100, 200, 400 and 800 µg/ml. Forty eight hr after treatment all flasks were exposed with EMF (50 Hz, 0.004 T). Then total RNA was extracted and cDNA was synthesized using specific primer. Synthesized products were analyzed by Real Time PCR to determine expression level of VEGFR₂. Data were analyzed by SPSS (ANOVA & Tukey).

Results: Critical inhibitory effect on VEGFR₂ gene expression was 20% at 400 µg/ml. Synergic use of EMF and saffron extract showed most reduction (38%) at 100 µg/ml. On the other hand synergic use of 200, 400 and 800 µg/ml saffron aqua extract and EMF decline noticeably the VEGFR₂ level of gene expression to 29, 35 and 36%, respectively. EMF itself also reduced VEGFR₂ up to 25% in comparison with control group which is remarkable at p<0.001.

Conclusion: Results indicate a decrease in the expression of vascular endothelial growth factor receptor in the treated samples with saffron extract compared to control. This reduction in VEGFR₂ level induced by synergic treatment of saffron and EMF which reveals induction of inhibitory effects of saffron on angiogenesis and could be also considered as a promising chemotherapeutic agent in breast cancer treatment.

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Introduction

Tumor angiogenesis which is related to Folkman's hypothesis explains about the growth of solid tumors as a result of blood vessel development. Also, it is regulated by expression of specific mediators which initiate a cascade of events leading to the formation of new micro vessels¹. At least five VEGF-related ligands are identified. Among these types, Vasular Endothelial Growth Factor-A (VEGF-A) is thought to be the most important angiogenesis simulator which is a hypoxia regulated gene². The biological effects of VEGF binding to three receptor tyrosine kinases, VEGFR-1, VEGFR-2, and VEGFR-3 are fairly well known. Research shows that VEGFR-2 is the primary receptor involved in angiogenesis, and its activation is considered critical in endothelial cell prolifera-

tion and survival³. The search for antiangiogenic strategies in cancer treatment indicated that targeting VEGF-A/VEGFR2 pathway resulted in significant inhibition of neovascularization and tumor growth⁴.

Saffron, the stigma of *Crocus sativus L.* (Iridaceae), currently used as spice, is the source of food additives and colorants and a component of traditional medicines⁵. This biomedical herb contains many constituents such as crocetin, picrocrocin and volatile compounds including safranal, crocin and crocetin^{6,7}. Saffron was evaluated for its pharmacological activities such as anticancer and antitumor and its constituents showed antioxidant activity in different organs such as muscle, kidney and hippocampus. Recent studies demon-

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strated the antitumor properties of saffron both *in vitro* and *in vivo*⁸. However, use of more effective treatments with less thread was considered by researchers during the recent years. One of these methods is using the electromagnetic field. Results of different studies showed different effects on organisms according to type of electromagnetic field, amplitude, frequency and time of exposure. Several mechanisms, both thermal and nonthermal, are well established by which electromagnetic fields can interact with biological systems⁹. It is also shown that static magnetic fields impair angiogenesis and growth of solid tumors *in vivo*. Furthermore, DNA, cell membrane and microtubules could be affected by EMF¹⁰. Therefore, the aim of this study was to investigate the synergic effect of EMF and saffron extract on VEGFR₂ expression in MCF₇ cell line. The study investigated whether 0.04 T electromagnetic field could increase inhibitory effects of saffron on VEGFR₂ expression in MCF7 cell line.

Materials and Methods

Saffron sample preparation

Original Iranian saffron (*Crocus sativus L.*) which is widely grown and gathered in autumn in south of Khorasan province was purchased from Novin Zaferan Co. (Mashhad, Iran). The plant was identified by a plant taxonomist from the Herbarium Division of the College of Ferdowsi University. The stigma's aqueous extract was prepared as follows: 3 g of dried stigmas was extracted with 250 ml of sterile distilled water by Soxhlet apparatus. The mixture was transferred to rotary to remove water. In order to dry the extract, lyophilization was done by using freeze dryer.

Cell culture

MCF7 cells were obtained from Pasteur Institute. Cells were cultured in RPMI medium (Biosera, Iran) with 10% fetal bovine serum (Gibco, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma, France) and also 1 ml L-glutamine (Sigma, France) and incubated at 37 °C with 5% CO₂. Twenty four hr after cell culture and after being confident about cell adhesion to flask, cells were treated with aqueous extract of saffron at concentrations of 100, 200, 400 and 800 µgm/ml. Then to evaluate the viability, Trypan blue (Sigma, France) test was used and the pictures were captured with invert microscope (Dinocapre) with a digital camera for 5 days to investigate cell morphological changes.

Exposing to EMF

After 48 hr of successful cell culture and treatment, samples prepared for testing the synergic effect were exposed to EMF. Flask was placed in 50 Hz, 0.04 T EMF for 1 hr (made in Islamic Azad University of Mashhad, Iran).

RNA extraction

RNA was extracted by total RNA purification kit (Bioscience, Germany). After 48 hr of treatment, total RNA was purified and stored at -20°C until cDNA synthesis. The amount of RNA Nano-drap was measured with wavelengths 260, 280 and 320 nm and data were obtained and analyzed. These data indicated the concentration of the extracted RNA which was used for cDNA synthesis. RNA concentration=(OD 260-OD 320)×40 ×100.

cDNA synthesis

cDNA was synthesized by Bioneer Kit (Korea) and the temperatures for synthesis are shown in table 1.

Synthesis of primers

The sequences of genes were received from NCBI site and the bioinformatic validation of RT-PCR primers were done by PRIMER BLAST and delivered to Bioneer Company (Korea). Primers were designed according to table 2.

Evaluation of gene expression

Process of real time PCR to study gene expression was done by Applied Biosystems according to the protocol of Bioneer kit (Korea). Based on the protocol, firstly, Master Mix was prepared and added to strip cap microtube and then the cDNA was added to it. The temperatures were determined according to Melting Temperature (T_m) of designed primers and the characteristics of different phases in polymerase chain reaction.

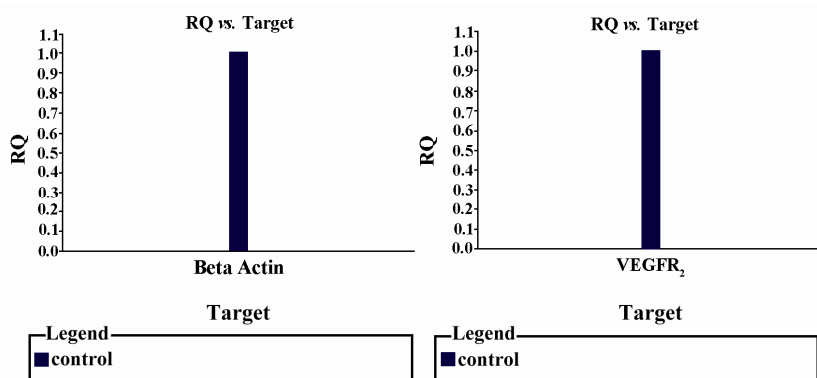
Gene expression levels of VEGFR₂ in samples treated with aqueous extract of saffron and the control samples were analyzed by SPSS (V.16). A p-value of less than 0.05 and CI (Confidence interval) of 95% were considered significant. In this project, we used relative quantities which were based on expression of target gene to the reference gene via comparing the target gene efficiency with control sample and also using their threshold cycle (CT). The primers and the temperature of

Table 1. The temperature required for the synthesis of cDNA

Step	Temperature	Time (min)
Primer annealing	T _m of specific primer	1
cDNA synthesis	42-70°C	10-60
Heat inactivation	95°C	5

Table 2. Sequences of genes

Gene	Forward 5'→3'	Reverse 5'→3'	TM	Chromosomal location
β -Actin	CCC GCC GCC AGC TCA CCA TGG	AAG GTC TCA AAC ATG ATC TGG GTC	64.5	7p22
Vascular endothelial growth factor receptor	TAT GTC TAT GTT CAA GAT TAC	AAG TTT CTT ATG CTG ATG CTT	42.9	4q11-q12

Figure 1. Housekeeping gene (β -Actin) expression in comparison with target gene ($VEGFR_2$)

binding primer were essential factors in the optimization of real time PCR reaction. So conditions of reaction were optimized and no nonspecific products were produced. This optimized condition observed by mono peak in melting curve and also electrophoresis in agar gel by looking single sharp band. The mean \pm SEM was determined for each study group. Data were analyzed by ANOVA and Tukey multiple comparison. The $p < 0.05$ was considered statistically significant.

Results

Since the PCR reaction efficiency between the target gene and the housekeeping gene (β -Actin) was the same (Figure 1), the comparative (CT) was used in this project. Amplification curve of samples treated with aqueous extract of saffron was obtained as well. Gene expression studies showed a significant reduction ($p < 0.05$) in gene expression levels of $VEGFR_2$ treated with concentrations of 100, 200, 400 and 800 $\mu\text{g/ml}$ in comparison with the control group.

Data analysis showed that inhibitory effects of

saffron extract in concentrations of 100, 200, 400 and 800 $\mu\text{g/ml}$ on $VEGFR_2$ gene expression were 16, 8, 20 and 18%, respectively in MCF7 cell line in comparison with the control group ($p < 0.05$). As data indicate, significant inhibitory effect on gene expression of $VEGFR_2$ was 20% in 400 $\mu\text{g/ml}$ of saffron extract ($p < 0.001$). EMF itself could reduce $VEGFR_2$ up to 25% in comparison the with control group ($p < 0.001$).

Synergic use of EMF and saffron extract resulted in most amount of reduction in concentration of 100 $\mu\text{g/ml}$ with inhibitory effect of 38% on $VEGFR_2$ level ($p = 0.001$). Moreover, the synergic use of saffron aqua extract in concentrations of 200, 400 and 800 $\mu\text{g/ml}$ and in electromagnetic field of 400 Gauss noticeably reduced $VEGFR_2$ level of gene expression to 29, 35 and 36%, respectively ($p < 0.001$). The difference between concentrations of 400 and 800 $\mu\text{g/ml}$ was not significant ($p > 0.05$). Figure 2 represents the level of $VEGFR_2$ gene expression in comparison with the control group after being normalized to β -Actin. It was also examined whether the CT variance was scaled

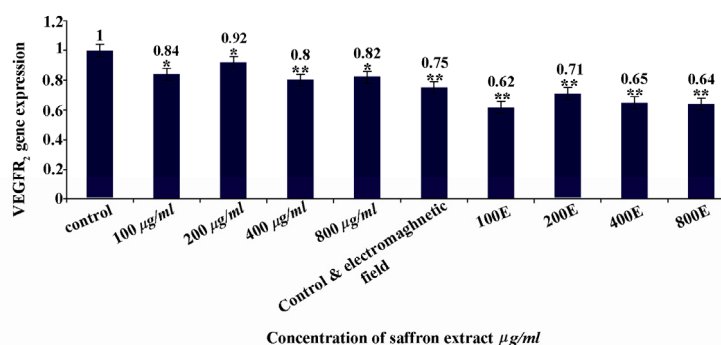


Figure 2. $VEGFR_2$ gene expression in samples treated with saffron aqueous extract in different concentrations and samples treated with saffron aqueous extract $\mu\text{g/ml}$ exposed to electromagnetic field for one hr. * $p < 0.05$, ** $p < 0.001$

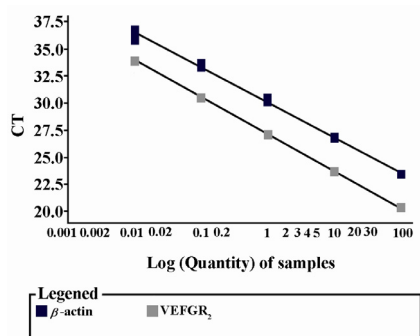


Figure 3. CT scaling down by using standard dilution samples with quantities of 100, 10, 0.1, and 0.01.

down by using standard dilution samples with quantities of 100, 10, 0.1, and 0.01 or not. Data analysis revealed that by dilution of samples CT changed and was scaled down. Acceptable data with high efficiency are represented in figure 3.

Discussion

In this research, we investigated synergic effect of low frequency (50 Hz) and 0.04 T intensity electromagnetic field and saffron extract on VEGFR₂ level in human breast cancer cells. It has been demonstrated that saffron's main components including crocin, crocetin and safranal could inhibit neuron erosion and depression ⁶.

Results of a research in 2009 revealed that the most important point in curing cancers with synthetic medication and chemotherapy is the acquisitive resistance of cells to chemical drugs because of high mutation and genetic variation in tumor cells comparing with other cells. Therefore their resistance to synthetic drugs is higher and angiogenesis inhibition with natural products such as saffron seems to be a useful and promising method for having less resistance ⁷.

There are several mechanisms for the antitumor effect of saffron and its components, including inhibition of nucleic acid, scavenging free radical, its effect on the expression of topoisomerase 2, induction of programmed cell death. Furthermore, it could reduce the possibility of developing cancer, decline the rate of tumor cells and significantly increase the lifespan of animals ¹¹. It was indicated that saffron in different tumors, including leukemia, ovarian and breast carcinoma has an anticancer and selective cytotoxic effect on different malignant cells ⁵. Moreover, saffron had cancer-preventive and antigenotoxic potential. Therefore, it can be used in combination with chemotherapy since some properties in saffron have the potential against lipid peroxidation and at the same time increase the level of enzymatic an-

tioxidants like superoxide dismutase and catalase and non-enzymatic antioxidants such as liver glutathione ¹². In addition, safranal increases tissue oxygen which has sweeping effect on free radicals and can inhibit oxidative stress of genotoxic compounds and safranal has protective effect on lipid peroxidation. Also, angiogenesis shows direct relation with tissue oxygen and hypoxia is one of the most important simulators of angiogenesis. Therefore, increasing tissue oxygen accompanied with saffron treatment may define some part of antiangiogenic effect of this herb ¹³.

Results of another research represented that saffron has antitumor effects on Transitional Cell Carcinoma (TCC) cell line (related to bladder cancer) which is time and dose-dependent in the sense that in high concentrations, the percentage of vital cells declines dramatically ¹⁴. Results of these researches are consistent with the findings of the present project. Influential potential of saffron on the induction or inhibition of gene expression in few cases has been studied so far. However, Mousavi *et al* had studied the effect of saffron extract on the level of protein which was related to apoptosis such as Bax protein and influential enzymes like caspase in breast cancer cells. They represented that using the extract could reduce cell viability based on the dose applied ¹⁵. The obtained results on the induction of apoptosis in saffron extract justifies that it can have significant cytotoxic effects on hepatocarcinoma and cervix cells ¹⁶. These results coincided with our findings and cancer cell growth was inhibited by the extract in a concentration-dependent manner.

In this research, the reduction in the level of VEGFR₂ and decline in gene expression by saffron extract demonstrated the conformity in our findings with other data that saffron has cytotoxic and anticancer effects. EMF was applied as the treatment for some specific pathologic conditions like bone fractures, skin ulcers, and migraine ¹⁷. Delle *et al* showed that some important functions of human micro vascular endothelial cells (*in vitro*) like proliferation, migration and tube formation are increased under the influence of EMF (1 mT, 50 Hz). Furthermore, they demonstrated that EMF increased the degree of endothelial cell proliferation and tubule formation and a significant increase in phosphorylation as well as the overall expression of VEGF receptor 2 was observed ¹⁸. Chen *et al* found the inhibitory effects of nanosecond pulsed electric fields on survival of mice with hepatocellular carcinoma. Also they ob-

served the decrease in vascular endothelial growth factor expression and micro-vessel density and apoptosis induction besides inhibition of angiogenesis¹⁹. Obviously, time of exposure, amplitude, intensity and type of electromagnetic fields and genetic characteristics of samples which were used in different researches were not the same leading to various findings. In addition, Wang *et al* reported that gradient static magnetic fields might inhibit or prevent new blood vessel formation and could be helpful for the treatment of some diseases relevant to pathological angiogenesis²⁰. According to this research, we observed the inhibitory effects of EMF on angiogenesis by reduction of VEGFR₂ level in human breast cancer cells. Moreover, synergic use of both saffron extract and EMF could decline VEGFR₂ gene expression which may account for antitumor property of saffron and novel antiangiogenic characteristic of EMF.

Conclusion

The results of this study indicated the reduction of VEGFR₂ gene expression in breast cancer cells treated with saffron aqueous extract and electromagnetic field. The study revealed both the antiangiogenic potential of this medical herb and therapeutic effects of EMFs as a promising chemotherapeutic agent in treatment of breast cancer, and its potential on prevention of angiogenesis.

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