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Research Article

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The synergic effect of low-frequency electromagnetic field and saffron extract on MMP gene expression in MCF-7 cell line

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ABSTRACT

Researches indicated using low-frequency electromagnetic fields(EMF)induce tumor cellsapoptosis, inhibit angiogenesis, impeded proliferation of neoplastic cells. Since angiogenesis could be target for tumor therapy and regards to the fact that matrix-metalloproteinase(MMP) has great effect on angiogenesis process as an extracellular protease. The aim of this research, was investigating the synergic effects of saffron and EMF on MMP gene expression in MCF-7 cell line. Saffron extracted using freeze dryer. MCF-7 cells were grown in RPMI-1640 medium supplemented with %10FBS and incubated at37˚C with%5CO2. After 24h cells were treated by saffron at concentration of 100,200,400and800µg/ml. 48h after treatment all flasks exposed with electromagnetic field (50Hz,0.04T) then total RNA was extracted and cDNA was synthetized using specific primer. Synthetized products were analyzed by Real Time PCR to determine expression level of MMP. Data analysis revealed inhibitory effect of saffron at concentration of 100,200,400and 800µg/ml on MMP gene expression in compare with control group with 5%,18%,15%and11% respectively (P<0.05). Low-frequency electromagnetic field itself also could reduce MMP up to 8% in compare with control group which is remarkable at P<0.05. Synergic use of saffron and electromagnetic field had inhibitory effect on gene expressionwith23%,20%,19%and16%respectively (P<0.05). Results indicated decrease in the expression of MMP in the treated samples with both saffron and exposure by low frequency electromagnetic field compared to controls.

Keywords: Saffron, EMF, MMP, MCF-7, Breast Cancer

INTRODUCTION

Angiogenesis, sprouting of new blood vessels from the pre-existing ones, plays essential roles in many both biological process under physiological conditions such as ovulation, embryo development, wound healing and also in a variety of diseases including diabetic retinopathy, rheumatoid arthritis, cancers and metastasis [1].Angiogenesis is required for embryogenesis and many physiological and pathological conditions such as tumor growth [2]. Tumor angiogenesis which is related to Folkman's hypothesis explains about the growth of solid tumors as a result of blood vessel development [3].The process is mediated by variety of cellular factors and condition such as hypoxic condition, low intra tumor PH, hypoglycemia, stress and inflammation which cause vascular endothelial growth factor expression from VEGF/PDGF super gene family. VEGF firstly binds to transmembrane receptors with intracellular tyrosine kinase activity [4].

This activity leads to secretion of metalloproteinase from endothelial cells to digest and decomposition basement membrane in the region. In the next steps, matrix metalloproteinase (MMP) degrade extracellular matrix and start re-reconstruction produced. By the interaction of the Tie-2 with angiopoitine tube formation begins at a later stage,

EphB-ephrin B also regulates formation the tubes, finally smooth muscle cells and precyts added to inducing the stabilization of newly formed blood vessel structure [4,5].

Matrix metalloproteinases (MMPs) degrade and modify the extracellular matrix (ECM) as well as cell-ECM and cell-cell contacts, facilitating detachment of epithelial cells from the surrounding tissue MMPs play key functions in embryonic development and mammary gland branching morphogenesis, but they are also upregulated in breast cancer, where they stimulate tumorigenesis, cancer cell invasion and metastasis [6].

Numerous of anti-angiogenic agents with various mechanisms of action are currently under clinical development and this research focused on the most promising and advanced among them by using natural product and most phenomenon spice: saffron.Saffron (*Crocus sativus* Linn.) related to Iridaceae family, the well-known food adjunct and bio colorant with pharmacological activities such as anticancer, antidepressant, anticonvulsant and treatment of memory impairment [7]. Saffron and its constituents especially crocetin, picrocrocin and volatile compounds including safranal, crocins (glycosyl esters of crocetin) which are unusual water soluble carotenoids showed antioxidant activity in different organs as like as muscle skeletal, kidney and hippocampus. It has been suggested that the antioxidant activity of saffron compounds can protect nucleic acids from harmful chemicals [8]. Different studies indicated the anti-cancer properties of saffron both in vitro and in vivo [9-11]. Breast cancer, which is the most important health problem for women worldwide, globally, is the most common type of cancer cause mortality in women [12]. Breast cancer in Iranian women compared with Western women, occurs at least one decade earlier and in more advanced stages [13].

Rising of cancer resistance to ordinary therapies, cause more effort to figure out new era in cancer therapy with fewer side effects and resistance. On the other hand, use of modern effective treatments, with less threatens considered by researchers. One of these valuable methods is using the low frequency electromagnetic field. Results of different studies show different effects on organisms according to type of electromagnetic field, amplitude, frequency and time of exposure and also the genetics of samples [14]. Numerous mechanisms, both thermal and non-thermal maintain which electromagnetic fields can interact with biological systems for example low frequency electric fields interfere with cell membrane and could decrease stimulation threshold of it [15]. Many researches reveal that using electromagnetic fields with low frequency could effect on angiogenesis process [16, 17]. It is also shown that static magnetic fields impair angiogenesis and growth of solid tumors in vivo and cancer cells. DNA, cell membrane and microtubules could affect by electromagnetic field. Changes in gene expression, cell interaction and cellular response have been also affected by electromagnetic fields [18].

In addition one of the most practical uses of Real Time PCR is defining gene expression using methods such as quantitative relative which is one of the precise and particular ways to study different changes in gene expression. In this method (Relative Quantitative) reduction or increasing of gene expression is important which is compared with reference gene, this referenced gene is a house keeping gene with specific and individual character such as equal copy number in all cancerous cells, capability of expression in all body cell and not affected by treatment [19].

As regards to identification an effective strategy by using new agents from natural sources to treatment breast cancer, and since inhibition of matrix metalloproteinasecould be apotent target in some pathological angiogenesis conditionsuch as breast cancer, the aim of this study was to investigated the synergic effect of saffron aqueous extract and sinusoid bioelectromagnetic field (50 Hz and 0.04 T intensity) on level of MMP in human breast cancercell line (MCF-7).

EXPERIMENTAL SECTION

- **Saffron Sample Preparation:**

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

- **Cell Culture & Treatment:**

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. 24 hours after cell culture and insurance about cell adhesion to flask, cells were treated with aqueous extract of saffron at concentrations of 100, 200, 400 and 800 micrograms per ml. Then to evaluate the viability, Trypan blue (Sigma, France) test was used and pictures

captured using a digital camera (Canon, Japan)with invert microscope (Biomed, Korea) for 5 days to investigate cell morphological changes.

- **Exposing with Bio electromagnetic Fields:**

After 48h of successful cell culture and treatment, samples which test synergic effect expose to EMF. Flask placed in 50 Hz,0.04 T EMF for 1h. . In order to generate required electromagnetic field, particular circuit for generating an electromagnetic field with 400 gauss (0.04 T) intensity was used (designed and made in Research Laboratory of Developmental Biology, Islamic Azad University of Mashhad, Iran,). The circuit includes coil, 3 Rheostat, capacitor and ammeter. To make bobbin coil, around the PVC (poly venial chloride) pipe adequate amount of copper wire according to the calculation of required electromagnetic field with equation of $B=\mu nI$ was wrapped. (B=intensity of electromagnetic field, $\mu=4\pi\times10$, n=the number of rounds per unite length, I=current intensity). To ensure the accuracy of electromagnetic field intensity gauss meter was used to control and calibrate.

- **RNA Extraction:**

RNA was extracted by total RNA purification kit (Bioscience, Germany). After 48 hours of treatment total RNA was purified and was stored at -20°C until cDNA synthesis. To measure amount of RNA Nanodrap used by wavelengths 260, 280 and 320 nm and data were measured and analyzed. These data indicate that the concentration of the extracted RNA which used for cDNA synthesis.

RNA concentration = (OD 260 - OD 320) * 40 * 100

- **cDNA Synthesis**

cDNA was synthesised by Bioneer Kit (Korea), temperature of synthesis was according to Table 1.

Table 1: The temperature required for the synthesis of cDNA

Step	Temperature	Time
Primer annealing	Tm of specific primer	1 min
cDNA synthesis	42-70 °C	10 – 60 min
Heat inactivation	95 °C	5 min

- **Synthesis of Primers:**

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

Table 2: Sequences of genes

Gene	Forward 5'→3'	Reverse 5'→3'	Chromosomal location
beta-actin	CCC GCC GCC AGC TCA CCA TGG	AAG GTC TCA AAC ATG ATC TGG GTC	7p22
Matrix metalloproteinase	CTG CAT CCT CAG CAG GTTG	GTC TCG GAT AGT CTT TAT CC	1q

- **Evaluation of Gene Expression Using Real Time PCR:**

Process of real time PCR to study gene expression was done according to the protocol by Bioneer kit (South Korea) by Applied Biosystem. According to the protocol Master Mix firstly was prepared and added to strip cap microtube then the cDNA was added to it. Application temperature according Tm of designed primers and the characteristics of the different phases in polymerase chain reaction were determined.

Gene expression levels of MMP in samples exposed low frequency electromagnetic field in comparison with control samples were analyzed by SPSS (V.16). P-value of less than 0.05 and CI (Confidence Interval) 95% was accepted as significant. In this project we use relative quantitative which is based on expression of target gene to the reference gene via comparing the target gene efficiency with control sample and also using their Ct.The primer and the temperature of binding primer are essential factors in the optimization of Real Time PCR reaction. So condition of reaction optimized in which no nonspecific products produced. This optimized condition observed by mono peak in melting curve and also electrophoresis in agar gel by looking single sharp band (Figure 2). The mean ± SD were determined for each study group. Data were analyzed by ANOVA & Tukey multiple comparison procedure to calculate the significance. $P < 0.05$ value between study groups was taken as statistically significant.

RESULTS AND DISCUSSION

It was also examined whether the ct variance scale down by using dilution standard samples with quantity of 100, 10, 0.1, and 0.01 or not. Data analysis reveals that by dilution of samples ct changed and scale down. Acceptable data with high efficiency represented in figure 1.

Since the PCR reaction efficiency between the target gene and the housekeeping gene (beta-actin) was the same (Figure 2), the comparative threshold cycle (CT) was used in this project. Amplification curve of treated with aqueous extract of saffron is identified in.

Gene expression studies showed a significant reduction (P<0.05) in the gene expression levels of matrix metalloproteinase. Data analysis showed inhibitory effect of saffron extract at concentration 100, 200, 400 and 800µg/ml on MMP gene expression with 5%, 18%, 15% and 11% respectively in MCF-7 cell line in compare with control group (P<0.05). As data indicate significant inhibitory effect on gene expression of MMP was 18% in 200 µg/ml of saffron extract (P<0.001). Synergic use of bio-electromagnetic field (50Hz,0.04 T) and saffron extract reveals most reduction at concentration of 100µg/ml saffron extract with 23% on MMP level (P<0.001). On the other hand synergic use of 200, 400 and 800µg/ml saffron aqua extract and 400 gauss' electromagnetic field decline noticeably the matrix metalloproteinase level of gene expression to 20%, 19%, and 16% respectively (P<0.001). The difference between 200 and 400µg/ml was not noticeable (P>0.05).

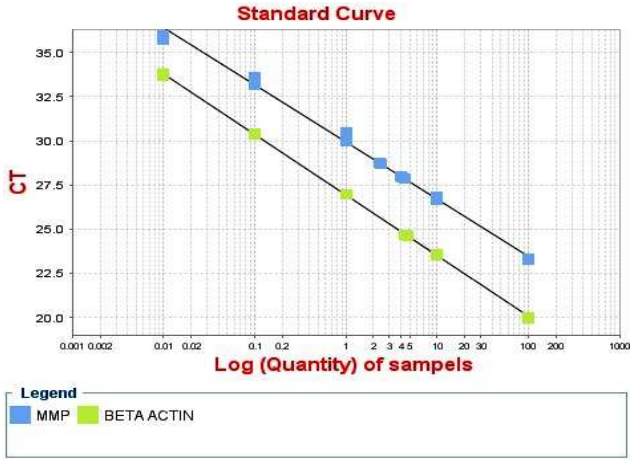

Figure 1: ct scale down by using dilution standard samples with quantity of 100, 10, 0.1, and 0.01

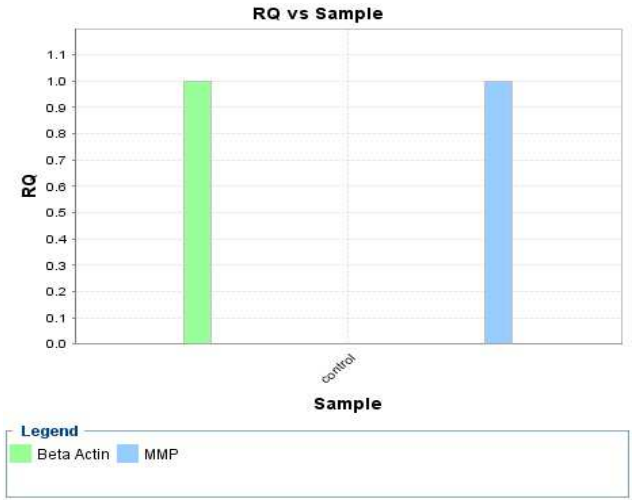

Figure 2: Housekeeping gene (beta-actin) expression comparing with target gene (MMP)

In this research, we investigated synergic effect of low frequency (50 Hz) and 0.04 T intensity electromagnetic field and saffron extract on matrix metalloproteinase level in human breast cancer cells.

Since extracellular proteolysis mediates tissue homeostasis and in cancer, altered proteolysis leads to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis, so the matrix metalloproteinases (MMPs) represent the most prominent family of proteinases associated with tumorigenesis and while MMPs regulate signaling pathways that control cell growth, inflammation, or angiogenesis study MMP function may be new approaches to cancer therapy [20]. Previously it was revealed that inhibition of matrix metalloproteinase with two murine models could lead to decrease angiogenesis and MMPs may be a target in cancer treatment and inhibition of metastasis [21-24]. Another research in 2009 was indicated EGFR activation lead to enhanced matrix

metalloproteinase expression and/or function, and suggest that modulating the expression or activity of the EGFR and/or matrix metalloproteinases offers opportunity for targeted intervention in patients with metastatic disease [25]. It has been demonstrated that saffron`s main component such as crocin, crocetin and saffranal could inhibit neurons erosion and depression [8]. It is indicated that saffron in different tumors, including leukemia, ovarian and breast carcinoma had an anticancer and selective cytotoxtic effect on different malignant cells [7]. Results of a research in 2008 represented that saffron have antitumor effects on TCC cell line (related to bladder cancer) in the time and dose dependent manner in the way that in high concentration the percentage of cells vital decline dramatically [26]. 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 and his colleague had studied the effect of saffron extract on the level of protein which were related to apoptosis such as bax protein and enzyme as influential as caspase in breast cancer cells and represent that using extract could reduce cell viability dose dependently [27]. The results on induction of apoptosis with saffron extract depicted that extract on hepatocarcinoma and cervix cells had significant cytotoxic effects [28]. These results were coincided with our findings and cancer cell growth inhibited by the extract on concentration-dependent manner.There are several mechanisms for the antitumor effect of saffron and its components, including inhibition of nucleic acid, scavenging free radical, effect on the expression of topoisomerase 2, induction of programmed cell death, furthermore it could reduce cancer, decline the rate of tumor cell and significantly increase the lifelong of animal [29]. Moreover, saffron had cancer-preventive and anti genotoxic potential, so it would be used in combination with chemotherapy, a part from that saffron shows reduction potential against lipid peroxidation and at the same time increase the enzymatic antioxidants like superoxide dismutase and catalase and non-enzymatic antioxidants such as liver glutathione regenerated [30]. In addition safranal increases tissue oxygen which have sweeping effect on free radicals and could inhibit oxidative stress of genotoxic compounds, safranal also had protective effect on lipid peroxidation; Since angiogenesis shows direct relation with tissue oxygen and hypoxia is one of the most important simulator of angiogenesis, so increasing tissue oxygen accompanied with saffron treatment may define some part of anti angiogenic effect of this herb [31]. In this research saffron extract can reduce the level of MMP and gene expression decline which shows conformity with other data that reveals cytotoxic and anticancer effect of saffron.

Some research showed exposure with electromagnetic field could lead to change cells' functional processes, induces different responses in living organisms such as effect on cell proliferation and differentiation, disorder in cell cycle and intracellular interactions, induction of planned death, DNA transcription, gene expression, cellular communications and free radical production [32]. While low frequency electromagnetic fields applied as treatment for some specific pathologic conditions like bone fractures, skin ulcers, migraines [33], according to Miyakoshi, 2006, electromagnetic fields induce noticeable DNA damages [34]. Funk, in 2006 also shows electromagnetic fields could reduce cAMP in bone cell culture up to 80% which is one of the most important inhibitory cell proliferation agents [35]. Since each cell have some electric parameter which cause ion charge in cell membrane and cancerous cell had a depolarize ruined membrane with permeability to water and Na^+ which lead to anaerobic respiration, hypoxia stress and releasing angiogenic factors, angiogenesis and metastasis. Some researches reveals in pico Tesla range electromagnetic field could be helpful in wound healing and reduce inflammation [36]. Another study reported reduction of angiogenesis in breast adeno carcinoma cells treated with 10, 15 and 20T electromagnetic fields [37]. Okana showed that 10 and 15 mT electromagnetic fields reduce angiogenesis in rats with experimental hypertension. He also showed that 120 mT static magnetic field (SMF) noticeably could reversed the inhibitory effects of TGF-1 on in vitro arteriogenesis, he suggested that SMF could have the potential to modify tubular formation, depending on the origin of the cells and the experimental conditions, including angiogenesis inhibitors or stimulators in the medium used for incubation, field intensity, localization of exposure, exposure duration and heterogeneous or homogeneous magnetic fields [38]. Delle showed some important functions of human micro vascular endothelial cells (in vitro), like proliferation, migration and tube formation are increased under the influence of a sinusoidal electromagnetic field (1 mT, 50 Hz), and the organization of the actin and focal adhesion inside the cell, he shows extremely low frequency sinusoidal electromagnetic fields increased the degree of endothelial cell proliferation and tubule formation and western blot analysis in their research revealed a significant increase in phosphorylation as well as the overall expression of VEGF R 2 [39]. Another research done by Chen and his colleague in 2012, revealed inhibitory effects of nanosecond pulsed electric fields on survival of mice with hepatocellular carcinoma with decreases in vascular endothelial growth factor expression and micro-vessel density andapoptosis induction and inhibition of angiogenesis [40]. It is also represented that the synergistic combinations of pulse electromagnetic fields (PEMF) or sinusoidal electromagnetic fields (SEMF) with hyperthermia (41.5°C) and/or cancerostatic agents presented in the tables for cells and mice offer a basis for further development of an adjuvant treatment for patients suffering from malignant tumors and metastases [16]. In addition Wang in 2009 reported that gradient static magnetic fields might inhibit or prevent new blood vessels formation and could be helpful for the treatment of some diseases relevant to pathological angiogenesis [41]. Obviously time of exposure, amplitude, intensity and type of electromagnetic fields and genetic of samples which used in different research were not same which cause different results.

CONCLUSION

The results of this study indicate reduction on MMP gene expression in breast cancer cells treated with the saffron aqueous extract and electromagnetic field which showed both the anti-angiogenic potential of this medical herb and therapeutic effects bio electromagnetic field as a promising chemotherapeutic agent in treatment of breast cancer, and its potential on prevention angiogenesis.

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