The effect of consumption of nettle extract and endurance training on the gene expression of IFN-Y and Endostatin in melanoma cancer in the liver tissue of mice

Running title: consumption of nettle extract and endurance training on the gene expression of IFN-Y and Endostatin in melanoma cancer

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Abstract

Background: Melanoma is an aggressive and malignant cancer that results from the transformation of pigment-producing melanocytes. This study aimed to investigate the effect of nettle extract consumption and aerobic exercise on the gene expression of IFN-γ and Endostatin in melanoma cancer in the liver tissue of mice.

Methods: Twenty male BALB/c mice were randomly divided into four groups, including control, endurance, nettle, and endurance+nettle. The training program included running on a treadmill for 30 minutes at a speed of 16 meters per minute. One meter per minute was added every week until it reached 22 meters per minute in the eighth week. Melanoma cells were induced subcutaneously on the left side of the mice. The experimental group consumed 30 mg/kg /day of nettle ethanol extract orally for 8 weeks. RT PCR was used to measure the expression of IFN-γ and Endostatin genes.

Results: IFN-γ gene expression in the experimental groups was not different from the control group, while the level of Endostatin was significantly reduced ($P = 0.142$, $P < 0.001$, respectively). IFN-γ gene expression levels in the experimental groups increased compared to the control group, but did not reach a significant level. Also, Endostatin gene expression levels in training and combination groups were significantly reduced compared to the control group ($P = 0.022$, $P < 0.001$, respectively).

Conclusion: The results showed that endurance training with nettle extract may inhibit angiogenesis and capillary tissue formation in the tumor tissue of mice with melanoma cancer by increasing IFN-γ and decreasing Endostatin.

Keywords: Melanoma cancer, IFN-γ**,** Endostatin, Nettle extract, Endurance training

Introduction

Melanoma is a skin cancer caused by the malignancy of melanocytes. The incidence of melanoma is rapidly increasing worldwide that leading to public health problems .Primary extra cutaneous melanomas can be ocular, gastrointestinal, mucosal, genitourinary, and lymphatic. The relationship between exposure to ultraviolet (UV) radiation and the development of melanoma is acute and complex, and intermittent sun exposure strongly increases the risk of melanoma. It is the fifth most common type of cancer in men and the sixth most common type of cancer in women (1). Early detection of skin cancer greatly reduces mortality and morbidity .Treatment and follow-up with a physician for melanoma patients may differ due to the stage of the tumor and the initial lesion (2). Interferons (IFNs) are a group of pleiotropic effects of cytokines that play an important role in intercellular communication during innate and acquired immune responses and defense against viral and bacterial infections (3, 4,5). In the tumor microenvironment (TME), IFN-γ continuously regulates antitumor and antitumor immunity. IFN-γ acts as a Cytotoxic function and cytokine along with Granzyme B and Perforin, to initiate apoptosis in tumor cells (6). In the environment of inflamed tissue or tumor, secreted pro-inflammatory cytokines bind to their receptors on IFN-γ-producing cells (6-9).

In this context, Endostatin is considered as a new biomarker for melanoma cancer (10). Endostatin has been described as an inhibitor of tumor angiogenesis and is a proteolytic fragment of 183 amino acids produced from collagen XVIII precursor (11). Previous studies describe multiple roles of Endostatin in modulating endothelial cell behavior. For example, Endothelin induces endothelial cell apoptosis and acts as a regulator of tube formation and endothelial cell migration and growth. Therefore, Endothelin interferes with tumor proliferation by inhibiting the activity of tumor-stimulating growth factors (12). In addition, several studies have shown that Endostatin inhibits tumor angiogenesis and tumor metastasis by limiting blood supply to tumors. As a result, it deprives tumors of nutrients and is considered as a potential anticancer agent in the treatment of malignant tumors (13). Physical activity affects the risk of various types of cancer. Available evidence strongly supports the role of regular physical activity in reducing the risk of colon, breast, and endometrial cancers to a lesser extent, lung and pancreatic cancers. It is also suggested that a similar relationship may exist for other cancers (14). Of course, the effect of physical activity on the level of interferon has resulted in different results in different studies.

Zamani et al. (2017) showed in their research that moderate activity increases the production of interferon-γ and interleukin-12 in peripheral blood mononuclear cells (15). Wijraigawa and Radhika (2014) investigated the response of interferon-γ production to activity intensity. The results showed that plasma IFN-γ decreases with a moderate acute exercise session. But severe acute exercise causes a sharp decrease. Regular moderate exercise increases interferon-γ (16). Golzari et al. (2010) investigated the effect of combined exercises on the level of IFN-γ and IL-17 in blood plasma in women with multiple sclerosis.Combined exercise training decreased IFN-γ and IL-17 levels in plasma and peripheral blood mononuclear cells (17).Shakur et al. (2018) investigated the effect of activity on gamma interferon body fat and BMI in kidney transplant patients.The results showed that physical activity had no significant effect on gamma interferon levels (18).Also Go et al. (2004) investigated the effect of physical activity on Endostatin levels in healthy people.The results showed that circulating Endostatin can be significantly increased by exercise in proportion to maximal oxygen consumption in physiological conditions in healthy volunteers (19).

The use of medicinal plants for the prevention and treatment of some diseases has been the focus of traditional medicine specialists since ancient times (20,21). The nettle plant is rich in essential amino acids, several minerals such as calcium, iron, magnesium, and phosphorus, and also contains vitamins such as C, B and K. In addition, it contains Flavonoids that show

antioxidant activity in addition to the anti-inflammatory activity of its polysaccharides (22,23). For example, a study by Mohammadi et al showed that the dichloromethane extract of nettle leaves inhibited the growth and proliferation of human prostate cancer cells (PC3) after 48 hours of treatment (24). Although herbal medicines still lack strong evidence for their role in cancer treatment and patient recovery, their use has become popular among cancer patients worldwide. The use of herbal medicines is supported by a large body of evidence from clinical studies reporting the benefits of several herbal medicines in reducing chemotherapy-induced toxicities (25, 26). Considering the effectiveness of safe and low-cost interventions, regular exercise and the use of herbal supplements can make the physiological effects of these interventions more specific as a cost-effective method. As a result, this study aims to investigate the effect of endurance training and consumption of nettle extract on the gene expression of IFN-γ and Endostatin in melanoma cancer in the liver tissue of mice.

Method

The subjects of this research project were adult male BALB/c mice, 6-8 weeks old, with an average initial weight of 350-300 grams. After purchase, these animals were transferred to the laboratory animal breeding and maintenance center. This research, which is the result of a working group, was approved by the ethics code number IR.IAU.M.REC.1399.008 in the Islamic Azad University, Marvdasht branch. After entering the research environment and getting familiar with the new environment and how to work on the treadmill for two weeks, the animals were randomly divided into 4 groups: control, endurance, nettle, and endurance+nettle. All stages of keeping and killing mice were done according to the Animal Ethics Committee of the Neuroscience Research Center of Shahid Beheshti University. Since laboratory mice are very sensitive to respiratory diseases, therefore, proper ventilation was placed in the storage area to prevent the accumulation of ammonia from animal urine. Endurance training was done four days after the start of supplementation for six weeks, 5 sessions a week on a treadmill. Mice in the training group exercised on a treadmill for 10 to 15 minutes for 1 week at a speed of 10 m/min for 5 days. From the second week, the overload phase was applied for three weeks until the end of the fourth week. The overload phase was such that on each training day, 3 minutes were added to the activity time and 1 meter per minute to the treadmill speed. By the end of the fourth week, the speed of the treadmill reached 28 meters per minute for 60 minutes of activity. From the fourth to the sixth week, the stabilization phase continued for three weeks at a speed of 28 m/min and for one hour.

B16F10 cells were purchased from the Pasteur Institute of Iran. These cells were chosen due to the same cell type as the studied mouse species. The cells were cultured in M199 medium and when the cell density reached 80%, they were prepared for injection into mice. The number of living cells before injection was counted by trypan blue staining. On the study day, 106 melanoma cells were subcutaneously injected on the left side of the mice (27). To prepare nettle extract, some of the stems and leaves of the nettle plant are collected after being cut into small pieces and washed, then dried in the open air and made into powder with a machine.

In the present research, to prepare nettle extract, some of the stem and leaves of the nettle plant were collected after being cut into small pieces and washed, then dried in the open air and made into powder with a machine. Then, 60 grams of nettle plant powder was placed in a 2.5-liter beaker and 2 liters of distilled water were added to it and the beaker was placed on a special heater (model MR3001 K, Heidolphl, Germany). After boiling, the intended decoction was filtered with filter paper. Extraction inside the distillation apparatus was placed in a rotary vacuum (Laboratory 4003 made by Heidolf, Germany) with a temperature of 45 degrees Celsius and a vacuum pressure of 65 mbar and a speed of 20 rpm. To prepare the solution, the aqueous extract of the nettle plant was dissolved in distilled water and in order for it to dissolve completely and obtain a thin and smooth solution, we placed it inside the Falcon tube and on

Vortex mixer so that the obtained solution could easily pass through the insulin syringe. To prepare the desired extract, the above steps were repeated several times. The experimental groups received nettle extract for 6 weeks in the amount of 30 mg per kilogram of body weight per day.

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Real-time polymerase chain reaction (real-time PCR) is commonly used to measure gene expression. The first step in a real-time PCR reaction is the conversion of RNA to complementary DNA (cDNA) – this process is known as reverse transcription. The next step uses fluorescent reporters and a PCR reaction to amplify and detect specific genes. Quantitation of RNA by kitRNX-Plus (SinaClone; RN7713C), and it was extracted according to the manufacturer's instructions. Then RNA extracted by the nanodrop spectrophotometer (ND-1000; Thermo Sci., Newington, NH) was investigated. A part of the extracted RNA sample was electrophoresed on a 1% Agarose Gel, and after staining with Ethidium Bromide, the RNA quality was checked. To check gene expression first from RNA, cDNA was made, which is complementary to RNA and can be used for this field's real-time PCR. The cDNA Reverse Transcription Kit and Cell and Tissue RNA Isolation Kit were used with the brand of Kiagene Fanavar.

The real-time PCR technique was used by Rotor Gene 6000 (Corbett Research, Australia) for 40 cycles to check gene expression .Primers of 3 genes with 1 control or reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were designed and ordered for synthesis from Synaclon Company (Table 1). For PCR, 2x master mix buffer, forward and reverse primer combination, cDNA, and injection water were used. The resulting mixture was prepared in the amount of 10 microliters in a special vial of the Corbett machine, and then it was placed in the router of the machine .The level of mRNAs of each gene was calculated relative to the level of mRNAs of the GAPDH gene. Primer sequence specifications for each gene are in Table 1.

Table 1. Primer sequence specifications for each gene

Gene	Primer sequence (5'->3')	
	Forward	**Reverse**
INF-Y	GAGTGTGGAGACCATCAAGGAAG	CTCACACCTCTGGTAGTTCCTTC
Endostatin	GGGGAATTCCACAGCCACCGCGAC TTCCAG CGACTTCCAG	CCCCTTAAGGTGTCGGTGGCGC CTCAAGGTCGCTGAAGGTC
GAPDH	AAGTTCAACGGCACAGTCAAGG	TTCAAGTTGCCGTGTCAGTTCC

Statistical Analysis

Quantitative description of data was done using central dispersion indices such as mean and standard deviation, and the Shapiro-Wilk test was used to determine the normality of data distribution and Levine's test was used to check the homogeneity of variances. Also, the one-way analysis of variance method was used to check the significant changes in each of the

research variables between different groups. If a statistically significant difference was observed, Tukey's post hoc test was used in the ANOVA program to determine the location of the difference between groups. The significance level for all calculations was considered as $p<0.05$. All statistical operations were performed using SPSS version 20 software.

Results

The data obtained from the research variables for five groups are presented descriptively in the form of Table 1. The mean and standard deviation of IFN-γ and Endostatin gene expression levels in the different studied groups show that the lowest concentration of IFN-γ was observed in the control group and the highest levels were observed in the combined group. Also, the results show that the lowest concentration of Endostatin was observed in the combined group and the highest levels were observed in the control group.

Data analysis using one-way analysis of variance showed that the expression of the IFN-γ gene in the experimental groups was not different from the control group. While Endostatin level had a significant decrease ($P=0.142$, $P<0.001$, respectively). The expression values of the IFN-γ gene in the experimental groups were increasing compared to the control group, but did not reach a significant level. Also, the expression values of the Endostatin gene in the training and combination groups were significantly decreased compared to the control group ($P=0.022$, $P<0.001$, respectively) (Table 2 and Diagram, Table 3 and Diagram).

Table 2. Analysis of variance test results related to IFN-γ gene expression in different groups **(**Arbitrary units)

Variable		Sum of squares	Degree of freedom	Mean square	F-test	p
IFN-γ	Between groups	9.876	3	3.292	2.089	0.142
	Within group	25.214	16	1.576		
	Total	35.09	19	4.868		

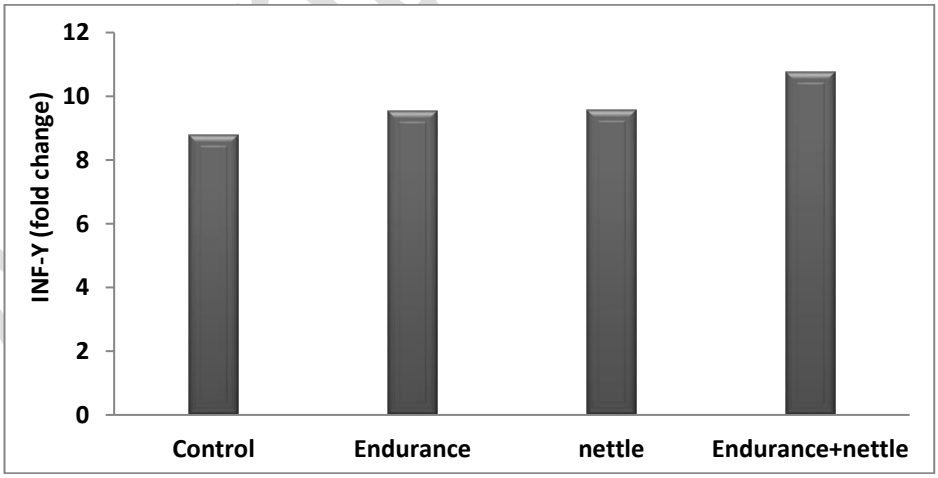

Diagram 1. IFN-γ gene expression changes in different research groups

Table 3. Analysis of variance test results related to Endostatin gene expression in different groups **(**Arbitrary units)

Variable		Sum of Squares	Degree of Freedom	Mean Square	F-test	p
Endostatin	Between groups	40.808	3	13.903	11.109	0.000
	Within group	19.592	16	1.224		
	Total	60.4	19	15.127		

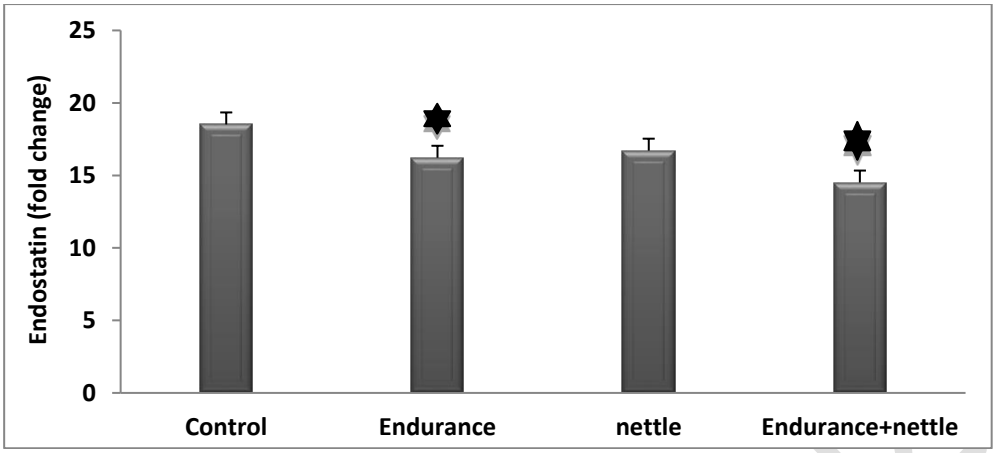

Diagram 2. Endostatin gene expression changes in different research groups
Difference with the control group (p=0.000)

Discussion

The results of the present research showed that nettle extract and endurance exercises have no significant effect on the level of **IFN-γ** gene expression in different groups.Although a non-significant statistical result was observed in the experimental groups compared to the control group. Interferon gamma (IFN-γ) is a pro-inflammatory cytokine produced by Cytotoxic T cells, CD4 T cells, and NK cells following immune system activation and inflammatory stimuli and acts against viral and bacterial infections (28).The results of the present study, which do not agree with the findings of several studies, including Golzari et al. the results showed that eight weeks of combined strength-endurance training in women with atherosclerosis led to a significant decrease in IFN-γ levels.most likely to related to other physiological parameters (17).Wang et al showed that 12 weeks of exercise training did not change IFN-γ levels and did not reduce inflammatory markers.Wang et al.'s findings support the findings of this research (29). However, Golzari et al.'s findings showed that eight weeks of concurrent training has beneficial anti-inflammatory effects by reducing the production of IFN-γ and IL-17. Physical activities increase the capacity of oxidative enzymes in muscles due to increased mitochondrial density.In addition, increasing the activity of the electron transport chain enzymes increases the activity of the enzymes involved in fat oxidation, especially the enzymes of the beta oxidation cycle and protein lipase (30). It seems that the difference of different reports about the response of IFN levels to sports activities originates from different factors that were different in different studies and thus brought contradictory results.Among these factors, we can mention the use of different protocols with different intensities in exercise in human or animal sample (31).It seems that the effect of immunosuppressive factors did not change IFN-γ in animal subjects with melanoma cancer.It seems that the lack of change in the concentration of IFN-γ is due to the intensity, duration and type of sports activities and consumption of of herbal medicines. (31)Also, the results of the research showed that endurance training and nettle extract have a decrease in the level of Endostatin gene expression in the experimental groups compared to the control group, which reached a significant level in the training and combination groups. Previous studies describe multiple roles of Endostatin in modulating endothelial cell behavior. For example, endothelin induces endothelial cell apoptosis and acts as a regulator of endothelial cell migration and growth (32). In addition, several studies have shown that Endostatin inhibits tumor angiogenesis and tumor metastasis by limiting blood supply to tumors. It can deprive tumors of nutrients and is considered as a potential anticancer agent in the treatment of malignant tumors. However, higher liver tissue Endostatin concentrations were found in various malignancies such as breast cancer, non-small-cell lung cancer, renal cell carcinoma and soft tissue sarcoma. In addition, Endostatin tissue levels are

significantly increased in patients with gastric cancer (33). In the present study, Endostatin levels were higher in the control group (melanoma cancer) than in other groups. Therefore, it is possible that an increase in tissue Endostatin level can be used as a biomarker for early detection and prediction of various types of cancer (10).Several studies showed a close relationship between increased Endostatin tissue levels and tumor stage in gastric cancer (34).However, other studies have shown conflicting results (10).Endostatin has various antitumor functions through the regulation of various receptors, including inhibition of angiogenesis and suppression of migration and invasion of tumor cells (35). Consequently, elevated liver Endostatin levels in advanced cancer may be attributed to production by cancerous tissues during tumor progression. (36). On the other hand a recent study provided strong arguments that Endostatin is a potent mediator of systemic inflammation and this could better explain association with advanced tumor stages. The results show that Endostatin tissue level may play a role in cancer progression. In accordance with our findings, Fujita et al also found that the serum level of Endostatin increased in gastric cancer patients. It also shows that Endostatin serum levels can be an important prognostic biomarker in predicting the survival of patients with gastric cancer (36). In confirmation of our findings, Feldman et al showed an increase in plasma Endostatin levels in colorectal patients with liver metastasis (37).Although Endostatin is a potent antiangiogenic agent in tumor lymph node involvement, circulating levels of Endostatin may not be sufficient to tip the angiogenic balance toward anti-angiogenesis. Previous research has shown that the effect of Endostatin on endothelial cells depends on the duration of its exposure.In addition, Endostatin concentration is important for optimal inhibitory effect. Celik et al showed that higher and lower doses of Endostatin have a lower inhibitory effect on lymph node involvement (38).Another reason for conflicting findings between studies in different cancers may be differences in angiogenic pathways in distinct tumor types (39).Plants contain several active chemical compounds at the same time and unlike chemical drugs, they can have synergistic effects and thus affect different aspects of disease pathology simultaneously. In other words, plant extracts rich in biologically active compounds can reduce the growth of cancer cells and induce apoptosis in them at the same time. This plants which lead to Tumor eradication by preventing angiogenesis and thus metastasis. Interactions of active compounds in plant extracts with tumors can give the immune system the opportunity to recognize and respond to the tumor cell. Plants contain several bioactive molecules capable of inducing cell protection and response to stresses such as antioxidant enzymes and apoptosis (40). In the present study, the levels of IFN-γ and Endostatin in the extract group did not change significantly compared to the control group.The lack of significant difference in the extract group may be due to the use of animal samples as well as the dosage and duration of extract consumption. Nettle plant extract may exert biological anticancer activities through various mechanisms, such as antioxidant and anti-mutagenic properties and induction or inhibition of key processes in cell metabolism (40).The most likely explanation for the significant anticancer effect of nettle plant is the known content of flavonoids.Among the bioactive molecules of nettle plant, flavonoids are polyphenolic compounds that are able to induce anticancer effects through various mechanisms such as antioxidant activity, apoptosis induction and inhibition of cell growth.In fact, several flavonoid-rich plants have disease-preventive and therapeutic properties, and consumption of flavonoid-rich vegetables and fruits in particular is associated with reduced cancer risk.Stinging nettle may be used as a bioactive nutrient in cancer therapy to prevent or reduce cancer without providing side effects of current anticancer treatments. However, further studies are needed to identify the pure bioactive molecules in this plant to better understand its multiple anticancer actions and explore these potentials in fighting human cancers. The results showed that endurance training with the consumption of nettle extract may inhibit angiogenesis in the tumor tissue of mice with melanoma cancer through the reduction of Endostatin. Also, Nettle

plant extract can exert anti-cancer activities through antioxidant properties and the anti-apoptotic.

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Conflict of interest

There is no conflict of interest for the research work, and all the costs of this article were paid personally by the doctoral student.

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Ethical statement

In this research, all the issues of the code of ethics were considered by the ethics committee.

Authors' participation

Mr. Vahid Zolghadriwrote the thesis and related articles, and his supervisors and advisors provided guidance and advice in writing all parts of the thesis and article.

Data availability statement

The data used in the research is available in the text of the student's thesis and in the central library of Ayatollah Amoli Branch of Islamic Azad University.

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