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The Effect of Eight Weeks of Aerobic Exercise on Gene Expression of Cytochrome C, Caspase 9 and Tumor Volume in Mice with Breast Cancer

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Abstract

Introduction: Breast cancer is a malignant tumor characterized by uncontrolled proliferation of cancerous cells by exposure to the risk of death. Therefore, this study aimed to investigate the effect of eight weeks of aerobic exercise on cytochrome C, caspase 9 and tumor volume in mice with breast cancer.

Methods: In this experimental study, 20 female balb/c mice (age: five to six weeks) were injected with MC4-L2 cancerous cells. They were then randomly assigned to two groups of 10 mice including experimental and control groups. Experimental group performed exercise for eight weeks (55-70% of the aerobic capacity of the mice). 48 hours after the last training session, the mice were sacrificed and their tumors tissue samples were removed and stored at -70 ° C and the level of factor expression of cytochrome C and caspase-9 of tumor tissue was measured by Real Time-PCR. Data were analyzed by SPSS21 using independent sample t- test ($p \leq 0.05$).

Results: There was a significant difference between the two groups of control and aerobic exercise in the cytochrome C ($P = 0.001$) and caspase 9 ($P = 0.039$), and the levels were higher in the exercise group than in the control group. Also, regarding tumor volume index, tumor volume in the exercise group was significantly less than that in the control group ($P = 0.001$).

Conclusion: In general, the results of this study confirm the positive role of eight weeks of aerobic exercise by stimulating upstream factors affecting the process of cancerous cell apoptosis.

Keywords: Cancer, Apoptosis, Exercise

Introduction

Breast cancer is one of the common diseases of women throughout the world. The risk of breast cancer is high in the presence of family history, especially in close relatives such as mother, sister or girl before premenopausal age. In breast cancer, like other cancers, the type of tumor (benign and malignant) or neoplasm determines the severity of the disease (1). Studies have shown that various molecular pathways, including inflammatory pathways, apoptosis, etc., contribute to cancer formation. Apoptosis is a planned cell death that is essential for maintaining the physiological balance between cell death and cell growth. Apoptosis is used in multicellular organisms to remove unwanted cells and maintain homeostasis during growth (2). The need to continue apoptosis depends on whether cell proliferation is beneficial to the living being. This is also related to apoptosis-inducing proteins (such as cytochrome C and caspases (cysteine-aspartic proteases) and inhibitor proteins (such as Bcl-2 (B-cell lymphoma 2)) (3). In general, increase in the levels of cytochrome C and caspases increases the rate of apoptosis and reducing them, results in the survival and proliferation of cancerous cells (3). Caspases play a central role in the onset of apoptosis. These enzymes prevent cellular wrinkling, and chromatinization and fragmentation of DNA. Caspase cascade response begins with the

activation of the startup caspases and transmits the message through the activation of the executable caspases. On the internal pathway, the genes express proteins that start the apoptosis (such as Bax, Fas, P53, etc.), and proteins that inhibit apoptosis (Bcl- 2 and Bcl-XL) and its consequence to the cell (death to survival) are dependent on the proportion of the genes expressed (3). Caspases coordinate the cell death pathway and, by decomposing their specific substrates, play an important role in promoting the expression of proteases as cell death. The pro-enzyme is activated by the breakdown by saline caspases. These enzymes have three main regions in the zymogen state: the second -N terminal, the large subunit containing the active site (containing cysteine) and the small sub-unit, including the C terminal. These three areas are separated by aspartate. Caspases are specific proteases, and decompose their substrates from a specific aspartate site that is compatible with caspase ability and can destroy proteins or activate other caspases. All caspases are activated by a protease degradation event. The active tetrameral caspase consists of two large subunits and two small subunits. Actin, vimentin, creatine and cadherin are suitable substrates for caspases (4). Cytochrome C is also a small hypo-protein found in the inner mitochondrial membrane. Unlike other cytochromes, this protein is strongly soluble in water and is one of the key components of the electron transfer chain, which is responsible for carrying an electron from complex 3 to complex 4 (cytochrome oxidase C). This protein has the potential for oxidation and regeneration, but it does not bind to oxygen. It is also one of the most important cofactors in the apoptosis process, which is essential for the activity of the ApaF-1 / caspase-9 protease complex (5). According to the stated points, measuring the interaction between the factors involved in the apoptosis process and controlling it in different conditions can help to find an effective way to improve the quality of patients with breast cancer. Treatment modalities may include radiotherapy, chemotherapy, and hormone therapy, depending on the stage of tumor growth and biological characteristics (such as the status of hormonal receptors). Despite the positive effects of these therapies on reducing mortality and the risk of cancer recurrence, they all have physical and psychological effects, such as decreased body function, fatigue, nausea, vomiting, and poor health. Evidence suggests that physical activity reduces some of these side effects (6). But a research study to directly evaluate the effects of aerobic exercise (considering the role of this practice in reducing cancer causing agents) on selected apoptosis agents in breast cancer patients (as in the present study) has not been carried out. Several studies have been carried out on the effects of physical activity on the reduction of inflammatory factors of the cancer and the reduction of tumor volume, often referring to the positive effect of physical activity on the reduction of cancer syndromes (7- 9). There are also sporadic studies with conflicting results in relation to the effect of different sports intensities on the apoptosis process (3, 10- 12). In two domestic investigations on the effect of aerobic exercise on breast cancer, the effect of aerobic exercise on Bcl-2 has been studied as an apoptotic stimulant in breast cancer patients and other relevant factors have not been studied (9). So far, less research has directly focused on the effects of aerobic exercise on the expression of cytochrome C and caspase 9 factors in mice with breast cancer. Considering the key role of apoptosis and other factors in inflammation, growth and metastasis of tumor cells and possible effects of exercises on the expression of these factors in cancer, a study of the effect of endurance exercise on the expression of the cytochrome C and caspase 9 genes in mice with breast cancer is necessary and it seems that the production and accumulation of information on this subject, can help the medical community on the one hand and the scientific community of sport on the other hand.

Methods

In this experimental study, 20 female balb/c mice (age: 5 to 6 weeks) were purchased as a statistical sample from the Iranian Institute of Pasteur. Mice were kept in separate cages with free access to water and food packs in accordance with the principles of animal care (NIH publication) in a 12-hour sleep and awakening cycle. The room temperature was maintained at between 22-24 ° C and 45% humidity. The animal's diet consisted of water and commonly-used mice food that was available ad libitum to the mice until the end

of the protocol. Mice were first placed in an animal house at the College of Physical Education and Sports Sciences of Tehran University for one week. During this time, all the mice got acquainted with the living conditions in the animal house and how to run on the treadmill. Mice then became infected with breast cancer by subcutaneous injection of cancerous cells. After tumor emergence, tumor volume was calculated every week (tumor volume was measured in two dimensions). The largest dimension of tumor was considered as the length (L) of the tumor, and the other dimension (at a 90-degree angle) was considered as width (W). Tumor volume was measured in both groups. To measure tumor volume, Jones *et al.*'s (2010) formula was used (13):

$$V=1/2(L^2\times W)$$

Then, 10 days after the onset of a cancerous tumor, the mice were randomly divided into two groups of 10 mice: tumor-exercise (TE) and tumor-rest (TR). The exercise hours for the exercise group were constant, and the exercise protocol was performed on 5 days in weeks, from 8:00 am to 10:00 am. In order to determine the aerobic power of the mice, an aerobic exercise test was first performed. The protocol for determining the aerobic capacity was as follows: After 5 minutes of warming up at a speed of 0.03 m/s (1.8 m/min), the treadmill speed increased once every three minutes; the maximum speed was when the mice couldn't run at a constant speed and couldn't run immediately after increasing speed (14). The exercises lasted for eight weeks. The exercises started in the first week at a speed of 16 m / min and eventually reached 22 m/min in the last two weeks (14) (55-70% of the aerobic capacity of the mice). After eight weeks of aerobic exercise protocol, following two days of rest, the mice were eventually sacrificed. Initially, the mice were anesthesized and sample of tumor issue was taken by surgical operation. Regarding tumor tissue, after freezing at -70 ° C by qRT-PCR method, the following stages in a molecular genetic laboratory were conducted: (1) Preparation of cytochrome C and caspase 9 samples; (2) Extracting RNA samples; (3) Investigation of optical absorption of samples by spectrophotometer; (4) Synthesis of cDNA from RNA; (5) Performing the Real Time PCR reaction; (6) Evaluation of cytochrome C and caspase 9 gene expressions in the experimental group and control group; (7) Data quantification using the method Δct. The sequences studied were as follows:

Cytochrome C

Forward:5'-AGTGTTCCCAGTGCCACACCG-3'
Reverse:5'-TCCTCTCCCCAGAATGATGCCTT-3'

Caspase 9

Forward:5'-GGGAGCAGAAAGACCATGGGT-3'
Reverse:5'-CGCAAACTTCTCACAGTCGATG-3'

Data were analyzed by SPSS21 using independent sample t- test ($\alpha \leq 0.05$).

Results

The mean and standard deviation of the body weight of the rats are presented in Table 1. Descriptive statistics and results of independent sample t-test are presented in Table 2. Results of independent sample t- test show that eight weeks of aerobic exercise revealed a significant difference between the two groups of control and aerobic exercise in the cytochrome C index ($P = 0.001$) in which the cytochrome C levels in the exercise group were more than the control group. Also, there was a significant difference between the two groups of control and aerobic exercise in the caspase 9 index ($P = 0.039$) in which the values of caspase 9 index in the exercise group were more than the control group. Regarding tumor volume, the results of independent sample t- test showed that there was a significant difference between the two groups of control and aerobic exercise in the tumor volume index ($P = 0.001$), in which tumor volume index values in the exercise group are less than the control group.

Discussion

The results of this study show the effect of eight weeks of aerobic exercise on the increase of selected factors affecting the apoptosis process and ultimately reducing tumor volume in the mice with breast cancer.

Table 1. Body weight (g) of rats in experimental and control groups

Group / Week	Experimental	Control
1	17.56±1.57	19.02±1.23
2	17.76±1.57	19.22±1.23
3	18.31±1.32	19.91±1.42
4	18.50±1.35	20.27±1.77
5	18.45±1.29	19.68±1.46
6	19.03±1.36	20.65±1.42
7	19.36±1.26	20.85±1.61
8	19.51±1.50	20.99±1.57
Mean	19.39	21.13
Standard deviation	1.39	1.53

Table 2. Results of independent sample t- test to investigate the effect of aerobic exercise on cytochrome C, caspase 9 and tumor volume

Variable	Group	Mean	Standard deviation	Levene's test	Independent sample t- test
Cytochrome C	Control	1.735	0.51990	0.16	0.001
	Experimental	4.639	0.78143		
Caspase 9	Control	8.17	1.34327	0.26	0.03
	Experimental	11.37	1.87689		
Tumor Volume	Control	135.82	52.71	0.07	0.001
	Experimental	59.84	32.48		

In general, the results of this study regarding the effect of aerobic exercise on reducing the tumor volume, are consistent with the results of Douglas *et al.* (2013), which indicated that physical activity increases the tumor growth index and increases the immune system to encourage an anti-tumor response (7); and Malinda *et al.* (2007) who stated that six weeks of aerobic exercise, five days a week, and each session one hour of exercise with 50% of maximum capacity reduced tumor volume and weight in the exercise group (8), as well as the research by Amani Shalmanzari *et al.* in 2014 who stated that eight weeks of aerobic exercise reduced the level of IL-6, endothelial growth factor of the vessels of the tumor volume (9). On the other hand, the results of this study regarding the effect of exercise on cell apoptosis are inconsistent with the results of Parko studies, which investigated the effect of eight weeks of regular exercise with low intensity (5 days a week) on cardiac and skeletal muscles of mice (the results of this study indicated an increase in Bcl-2 and Hsp70, a decrease in ApaF-1 and Bax, and a no significant change in caspase-3) (15) as well as the results of Fernandez's research that investigated the effect of 10 weeks of regular aerobic exercise on the apoptosis in male rats (the results of this study indicated an increase in Bcl-2 and a decrease in BAD and inhibition of apoptosis in general) (11). But the results of the present study were consistent with the results of Fanwaf study (which showed that immediately after one bout aerobic exercise, Bcl-2 levels decreased and Bax levels increased (3) and the Bieral (which indicated that after one bout extroverted aerobic exercise levels of Bax and caspase 3 increased and apoptosis was induced (10). The reason for contradictions can be attributed to the duration and period of the exercise as well as the health status of the subjects. Given the moderating role of exercising, these contradictions are not mind-boggling. The results of this study regarding the specific effect of aerobic exercise on the reduction of selected apoptosis factors in patients with breast cancer are consistent with the results of Amani *et al.*'s research (which stated that six weeks of aerobic exercise reduced inflammatory factors in the tumor, and decreased Bcl-2 expression

and increased apoptosis (9) and Amini *et al.* (who stated that eight weeks of aerobic exercise increased miR-15 and decreased BcL-2 and tumor growth proportion in the exercise group compared to the control group (16). According to the results of this study, aerobic exercise seems to reduce the cancer cells from the pathway of apoptosis, so that due to compatibility with eight weeks of aerobic exercise, definite upstream factors are activated that destroy mitochondrial membranes and release apoptotic molecules such as cytochrome C into cytosol. Cytochrome C activity will activate caspase 9, which ultimately increases apoptosis (2). One of the important aspects of mitochondrial biology is the role that this organel plays in apoptosis. Mitochondria are an integral part of the internal pathway of apoptosis and the location of many of the involved proteins in the early stages of this process (17). It seems that increased oxygen consumption and increased pulmonary ventilation and metabolism due to aerobic exercise sessions in the subjects of this study have led to an increase in the production of ROS within various mechanisms including arachidonic acid metabolism and sequential respiration activity from leukocytes and through oxidation of purine and pyrimidine bases, and especially guanine, have damaged the DNA and caused apoptosis of cancerous cells. Also, the significant amount of oxidants produced by exercise can also degrade DNA and directly stimulate apoptosis (3). On the other hand, probably due to the increase of ROS and toxic chemicals, as well as the stimulation of the production of certain apoptotic microRNAs such as miR-15 and miR-16 induced by aerobic exercise, release of Bcl-2 is inhibited, and instead, Bax, an important agent and apoptotic stimulant, is increased from mitochondria, both of which trigger release of cytochrome C from the space in the membrane into cancerous cells. The cytochrome C released by aerobic exercise, in addition to being the main actor in the process of activating the mitochondrial caspase cascade, interacts with mitochondrial phospholipid, namely cardiolipin. The result of this reaction is the formation of the cardiolipin-cytochrome C complex, which has catalyzed oxidation of cardiolipin in the presence of hydrogen peroxide. The accumulation of products of this oxidation in mitochondria has resulted in the release of pre-apoptotic factors into cytosol (18, 19). The release of cytochrome C released by aerobic exercise induces oligomerization of ApaF-1 and as an agent associated with the activator of apoptosis-1, which is normally inactive and passive in the cell, and in the process of apoptosis with mitochondrial cytochrome c release is activated, attaches to it and generates dATP, activates procaspase 9, activates caspase 9 and eventually caspase 3, and acts in the direction of apoptotic pathway, and this process by crushing and ultimately phagocytosis of the cancerous cell has reduced tumor volume (20- 23).

Conclusion

In general, the results of this study confirm the positive role of eight weeks of aerobic exercise in stimulating apoptosis in cancerous cells. Improvement of mitochondrial performance due to compatibility with aerobic exercise leads to inhibition of the upstream factors and, by decreasing its levels, increases the expression of apoptosis-inducing genes, including cytochrome C and caspase 9, and thus decreases cancerous cells and decreases tumor volume.

Ethical issues

Not applicable.

Authors' contributions

All authors equally contributed to the writing and revision of this paper.

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