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Original Article

The Effect of 4 Weeks Aerobic Exercise Training with Detraining Courses in Various Prevention Phases on BCl-2 and BAX Genes Expression and Proteins

Abstract

Background: The aim of this study was to investigate the effect of aerobic exercise with detraining in different phases of prevention on BCL2 Associated X (BAX) and B-cell lymphoma 2 (BCl-2) gene expression and proteins. **Methods:** For this purpose, 32 female Balb-c mice (18–20 g) were purchased and randomly assigned to primordial prevention (A), primary prevention (B), secondary prevention (C), and control (D). A group performed aerobic exercise for 4 weeks, after 4T1 cells injection detrained for 8 weeks. Group B performed aerobic exercise for 4 weeks immediately after injecting 4T1 cells and then detrained for 4 weeks. In C group, the 4T1 cells were first injected and did not perform any activity for 4 weeks, followed by 4 weeks of aerobic exercise. Forty-eight hours after the last training session and detraining courses, after anesthesia, sacrificing, and tissue removal, were performed. Reverse transcription polymerase chain reaction (RT PCR) was used to measure gene expression and Western blot (WB) was used to measure protein content. One-way Analysis of variance (ANOVA) test was used to analyze data. **Results:** The results showed that aerobic exercise in A, B, and C groups compared to D group reduced BCl-2 gene expression and protein and increased BAX gene expression and protein. **Conclusions:** Therefore, exercise can cause apoptosis in tumor cells by increasing pre-apoptotic factors and decreasing antiapoptotic factors in tumor cells, and consequently improving the disease status.

Keywords: *Aerobic exercise, apoptosis, detraining, primary prevention, primordial prevention, secondary prevention*

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Introduction

The increasing prevalence of cancer in recent years and its impact on various physical, mental, and social dimensions of human life has made it one of the main problems of the century.[1] The prevalence of the disease in developed countries varies between 1% and 2% and increases by approximately 5% each year in less developed countries.[2] Among the various types of cancer, breast cancer, which accounts for 23% of all cancers in women, is the most common cancer and the deadliest malignancy in women and one of the most worrying factors in women's health in the world.[3,4] In Iran, breast cancer is the most common malignancy among women[5] and accounts for about 32% of women's cancers.[6] According to the Cancer Registration System in Iran in 2009, about 49.4% of breast cancer cases occurred in the age group of 50 years and older, 31.2 in the age group of 40–49 years, and 19.4 in the age group of less than 40 years.[7] The most common age of onset in Iranian women is 47 years and one decade younger than in developed countries.[8] On the other hand, the International Agency for Research on Cancer has estimated that 25% of the causes of cancer are obesity or overweight and sedentary lifestyle.[9,10] Weight gain of 1.5 times normal increases the risk of breast cancer.[11,12] Overweight and high BMI during and after menopause significantly increase the risk of breast cancer.[11] This is due to an increase in estrogen from adipose tissue, which can inhibit apoptosis and increase cell proliferation.[11] Decreased apoptosis or its resistance plays an important role in carcinogenesis. There are many ways that a malignant cell can reduce apoptosis or resist apoptosis. In general, the mechanisms that occur in the escape of apoptosis can be broadly divided into (1) impaired balance of pre-apoptotic

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and antiapoptotic proteins, (2) decreased caspase function, and (3) impaired signal receptor signaling.[13] In fact, apoptosis is the ability to kill mutated cells that can lead to cancer. There are three ways to activate caspases, in which one of them is intrinsic (mitochondrial) pathway. This pathway is controlled by a group of proteins belonging to the Bcl-2 family. The Bcl-2 protein group consists of antiapoptotic proteins such as Bcl-2, Bcl-XL, and pre-apoptotic proteins such as BAX.[13] Bax protein is the initiator of apoptosis through the formation of mitochondrial membrane permeability.[14] In contrast, Bcl-2 has antiapoptotic function and cell preservation.[15,16] Activating the Bax expression prevents the Bcl-2 function.[16,17] The ratio of these two proteins can affect the presence or absence of apoptosis because of the increase in Bax/Bcl-2 ratio, which is considered a reliable cell indicator for apoptosis.[17,18] BcL2 plays a central role in the genetic program for the growth and survival of eukaryotic cells by inhibiting cell death.[19] Therefore, BcL2 suppressors can be used as a treatment for cancer by reducing the inhibitory effect of BcL2 on apoptosis.[20] There is ample evidence that women have regular physical activity, also postmenopausal women compared to premenopausal women, compared to women who are inactive, 10–20%, are less likely to develop breast cancer.[21] A recent report by Study Health Nurses on more than 95,000 women found that increased postmenopausal physical activity reduced the risk of breast cancer by 10%, which may be due to the effects of physical activity on body mass, hormones, and energy balance.[22] Several studies have shown that women who were physically active had a 24–67% overall mortality rate and a 50–53% lower risk of dying after breast cancer after women who had no physical activity. Regular exercise during adolescence and adulthood can help reduce the risk of breast cancer. Among breast cancer survivors, regular exercise can reduce the side effects of treatment (radiation therapy and chemotherapy) and increase the chances of survival.[23] On the other hand, due to the fact that fat is the storage of estrogen and the production of steroid hormones and obesity is one of the factors affecting the occurrence of various cancers, it seems that the effect of exercise on fat reduction can be effective in improving cancer.[24] Given the positive effects of exercise on breast cancer, the present study seeks to find an answer to the question of whether aerobic exercise in the primordial, primary, and secondary prevention phases can lead to the induction of apoptosis in cancer cells of Balb C mice? And does the phase of exercise intervention affect the possible changes in exercise activity on apoptosis in cancer cells?

Methods

Animals

Thirty-two Balb C rats (7–8 weeks old with an average body mass of 18–20 g) were purchased from the Pasteur Institute and transferred to Animal Laboratory of the Biological Sciences, University of Isfahan. For the physiological matching of mice, a 12-h dark-light period was observed. Room temperature was also maintained between 22 and 24°C, and 45% humidity. Animal feed was freely available to mice until the end of the protocol. After identifying the mice with the environment, they were divided into four groups (aerobic exercise in the preliminary prevention phase, aerobic exercise in the primary prevention phase, aerobic exercise in the secondary prevention phase, and control). It should be noted that at the end of the study, due to mortality, etc., the number of mice in each group decreased to 6. Approval ID: IR.UI.REC.1398.012,, Approval Date: 2019-06-30.

Research plan

The research plan is described in Table 1.

Cell culture

In this research, first 4T1 cell category was prepared from the cell bank of Isfahan University of Medical Sciences. Then, defreezing of the frozen cells was done, and after 24 h, the culture medium was replaced. The cells were cultured in the 1640 Roswell Park Memorial Institute (RPMI) medium with 10% serum of cow embryos in a CO_2 incubator. Then, 2 days later, to pass, first the top liquid was drained with a pasteur pipette and then the cell surface was washed with 1-ml phosphate-buffered saline (PBS) and the liquid was discharged with a pasteur pipette. The 2-ml trypsin was then poured into a flask containing the cells and placed in an incubator for 3–4 min. After ensuring that the cells separated from the bottom of the flask, the entire contents of the flask were poured into a Falcon 15 with a pasteur pipette. To counteract the effect of trypsin, a culture medium containing 10% Fetal Bovine Serum (FBS) was poured 2 times as much as trypsin (4 ml) in Falcon and placed in a centrifuge at 15,000 rpm for 4 min. Then, all the liquid on the cell plaque was discarded. One millimeter of culture medium was poured on the cell plaque and they were well pipetted. The cells were then counted on a lam under a microscope.

4T1 cell injection

To breast cancer creation, after anesthesia, two million 4T1 cells dissolved in 100 µl PBS were subcutaneously injected into the area next to the mice's right foot. About 3–4 weeks after the injection, the tumor under the skin of mice was completely palpable at the injection site.

Aerobic exercise protocol

To perform the exercise intervention, familiarization to treadmill was first performed for 2 weeks. After the familiarization, the main training protocol, which included 4-week courses, was performed 5 days a week. The aerobic exercise was consisted of 40 min of running on a rodent treadmill at 18 m/min per session.

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Table 1: Research plan

Groups	2 weeks	4 weeks	4 weeks	4 weeks	48 h after last section
Primordial phase	Introduction to treadmill	Aerobic exercise	Detraining	Detraining	Sacrifice and tumor tissue removal
Primary phase		Inactivity	Aerobic exercise	Detraining	
Secondary phase		Inactivity	Inactivity	Aerobic exercise	
Control	Inactivity	Inactivity	Inactivity	Inactivity	

Tumor volume measurement and tumor growth

Three to 4 weeks after cancer cell injection, after the tumor appears, the tumor volume was calculated each week. The Jones *et al*. Formula ($\pi/6$ ($L^2 \times W$)) was used to measure tumor volume.[25] Therefore, tumor volume was measured in two dimensions. The largest dimension of the tumor was considered as the length (L) of the tumor and the other dimension (at a 90° angle) was considered as the width (W). Tumor volume was measured in all four groups. Tumor growth was obtained by dividing the tumor volume in the final week by the tumor volume at the end of the eighth week.

Sacrifice of mice and tissue removal

Forty-eight hours after the last training session, after anesthesia with ketamine and xylazine, the mice were sacrificed and the tumor tissue was removed. After removing the central necrosis part, it was placed in the C-73 freezer.

Real-time PCR protocol

To evaluate the expression of BAX and BCL-2 genes, RNA extraction was first performed by ROUCHE Trizol solution. After spinning, the concentration of the sample was read using the nanodrop device. To prepare the first strand of cDNA from the total RNA extracted in the previous step, a kit with the TAKARA brand was used. This kit is based on the reverse transcriptase enzyme. A microgram of the synthesized cDNA was then injected into the real-time PCR reaction, which was performed with the help of TAKARA's Green Cyber Green Kit. Each pair of primers for each gene was designed using oligo7 software and synthesized by Roche. The β-actin was used as a housekeeping gene. The test results were analyzed by $2^{-\Delta\Delta CT}$ method and their fold change results were reported.

WB protocol

To determine Bcl-2 and Bax protein expressions, Western blot analysis was used. First homogenization of cells was performed on ice in 100 µl lysis buffer of Radio-Immunoprecipitation Assay (RIPA) (50 mM Tris–HCl (pH 8.0), 0.1% sodium dodecyl sulfate, 150 mM sodium chloride, 0.5% sodium deoxycholate, and 1.0% NP-40) and then the cells were centrifuged at 12,000*g* for 15 min at 4°C. After collecting the supernatant, the protein concentration was determined using the Bradford method using Bio-Rad commercial reagents (Bio-Rad Laboratories, CA, USA). After separating the proteins using electrophoresis on 12% SDS electrophoresis, the polyacrylamide gel was transferred to the pre-activated polyvinylidene fluoride membrane. Incubation of membranes in the blocker solution (bovine serum accepted manuscript albumin 1% in PBS plus 0.1% Tween-20) was performed for 2 h with a gentle vibration to prevent nonspecific bonding. Then, the blots were incubated with various primary rabbit antibodies (anti-Bcl-2 (1:500), (sc-492), anti-BAX (1:500), (sc-7480), and anti-β-actin (1:300), (sc-47778)) overnight at 4°C. After 4 times washing with PBS, the blots were finally incubated with horseradish peroxidase (HRP)-conjugated antirabbit secondary antibodies (mouse anti-rabbit IgG-HRP: sc-2357 and m-IgGκ BP-HRP: sc-516102; Santa Cruz, USA (1:5000)) for 1 h at room temperature. With the use of enhanced chemiluminescence and quantified by ImageJ software, a protein band emerged. β-Actin was also used as an internal loading control.

Data analysis

To analyze the research data, one-way analysis of variance and Tukey's follow-up test at a significance level of 0.05 were used.

Findings

For data natural distribution test, the Shapiro–Wilk test was used, and the results showed that the data distribution was normal ($P > 0.05$). The one-way ANOVA test was used to compare groups. The increase in tumor volume from the end of the eighth week to the end of the twelfth week. As can be seen in the diagram, the tumor volume has increased in four groups, but this increase is more in the control group than in other groups [Figure 1].

Table 2 shows that the mean and standard deviation also results from one-way analysis of tumor growth, BCL-2 gene expression, BAX gene expression, BCL-2 protein, and BAX protein in primordial prevention, primary prevention, secondary prevention, and control groups.

The results of the one-way ANOVA test showed that there was no significant difference between the groups in the growth rate of the tumor ($F_{3,20} = 2.47$, $P = 0.92$) [Figure 2].

However, there was a significant difference between the groups in the BCL-2 gene expression ($F_{3,20} = 111.53$, $P = 0.001$). After the Tukey post-hoc test, there was a significant difference between the groups of primordial prevention and control ($P = 0.001$), primary prevention and control ($P = 0.001$), and secondary prevention and

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control (P = 0.001) groups, but the difference between the other groups was not significant. Also, based on the one-way ANOVA test, the difference between groups in BCL-2 protein content was significant ($F_{3,20}$ = 5.90, P = 0.005). This significant difference between the primordial prevention and control (P = 0.011) and the secondary prevention and control (P = 0.007) groups was observed; but there was no significant difference between the other groups [Figure 3].

The results of the one-way ANOVA test showed that the difference between the groups in the expression of BAX gene was significant ($F_{3,20}$ = 6.14, P = 0.004). By examining the Tukey post-hoc test, this significant difference was observed between the primordial prevention and secondary prevention (P = 0.031) and the secondary prevention and control (P = 0.004) groups. But there was no significant difference between the other groups; also the results showed that the difference between the groups was significant in the expression of BAX protein ($F_{3,20}$ = 0.129, P = 0.001). This significant difference was observed between the primordial prevention and control (P = 0.001), primary prevention and control (P = 0.004), and secondary prevention and control (P = 0.005) groups. But there was no significant difference between the other groups [Figure 4].

Discussion

The aim of this study was to investigate the effect of 4 weeks of aerobic exercise in different phases on gene expression and BAX and BCL-2 protein and tumor growth.

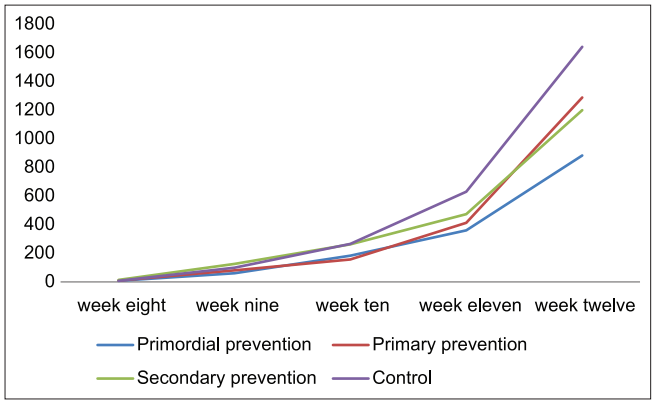

Figure 1: Mouse tumor volume, from the end of week 8 to the end of week 12 of study

The results of the present study showed that aerobic exercise training in different phases had a significant effect on gene expression and BCL-2 protein. These results were consistent with the results of De Lima *et al*.,[26] Kurdi *et al*.,[27] and Rafiei *et al*.[28] studies. Also, aerobic exercise training in different phases showed a significant effect on gene expression and BAX protein expression. These results were consistent with the results of Kurdi *et al*.,[27] Rafiei *et al*.,[28] and Alizadeh *et al*.[29] researches.

IL-6 in breast cancer and some other tumor cells has been reported to increase resistance to apoptosis by increasing BCL-2.[30] Because regular exercise lowers IL-6 levels,[27,31] one possible mechanism for Bcl-2 decrease could be IL-6 decrease. On the other hand, BCL-2 can reduce BAX in mitochondria by preventing the transfer of BAX from cytosol to mitochondria.[32] Therefore, IL-6 indirectly reduces BAX, and exercise increases BAX by reducing IL-6. Bcl-2 mRNA expression has also been shown to be reduced in anti-Mir-treated cells. MiR-21 may indirectly regulate Bcl-2 expression.[33] Accordingly, a possible explanation could be the repressive effects of anti-miR-21 gene expression with subsequent negative regulation of Bcl-2 expression. STAT3 can also modulate the expression of a number of transcriptional antiapoptotic proteins, such as Bcl-2.[34] Therefore, the effect of STAT3 on miR-21 may play an important role in controlling Bcl-2 expression. In the present study, exercise increases Bax protein in mice tumor tissue. Estrogen can inhibit apoptosis through dual responses, such as stimulation of Bcl-2 or suppression of Bax products.[35] There is growing evidence that physical

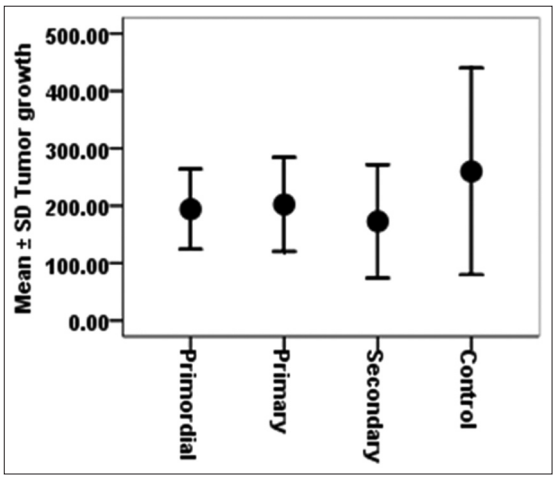

Figure 2: Mean and standard deviation of tumor growth rate in study groups

Table 2: ANOVA's one-way results of research variables in four groups

Variables	parameter	Primordial	Primary	Secondary	Control	*F*	*P*
Tumor growth	Mean±SD	193.9±35	202.2±41	172.8±49.4	259.8±90.1	2.47	0.092
BCL-2 gene expression	Mean±SD	0.48±0.15	0.32±0.15	0.04±0.02	1.7±0.26	111.53	0.001
BCL-2 protein	Mean±SD	0.69±0.19	0.82±0.18	0.67±0.16	1.00±0.00	5.90	0.005
BAX gene expression	Mean±SD	1.72±0.42	3.06±0.59	5.86±1.58	0.90±0.69	6.14	0.004
BAX protein	Mean±SD	2.29±0.65	2.07±0.39	2.56±0.57	1.00±0.00	9.01	0.001

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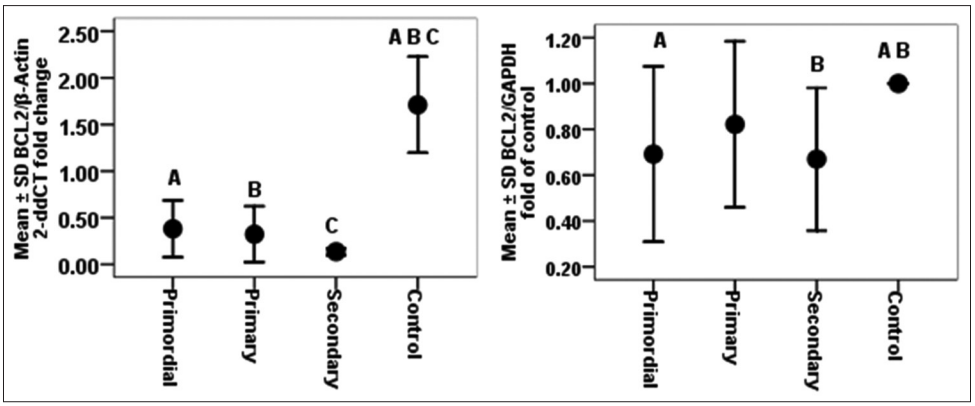

Figure 3: Average and standard deviation of BCL-2 gene and protein expression in study groups. A Significant difference between primordial phase and control (gene expression); A: significant difference between primordial phase and control (protein). B: Significant difference between primary phase and control (gene expression); B: significant difference between secondary phase and control (protein). C: Significant difference between secondary phase and control (gene expression)

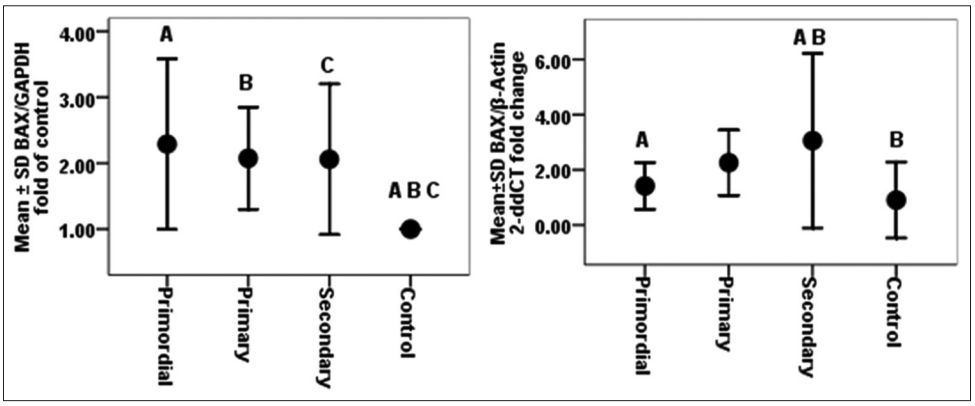

Figure 4: Average and standard deviation of BAX gene and protein expression in study groups . A: Significant difference between primordial phase and control (protein); A: significant difference between primordial and secondary phases (gene expression). B: Significant difference between primary phase and control (protein); B: significant difference between secondary phase and control (gene expression). C: Significant difference between secondary phase and control (protein)

activity modulates estrogen-induced breast cancer in several acceptable biological mechanisms,[36] so exercise by lowering estrogen levels can lead to increased apoptosis by increasing BAX and decreasing BCL-2. It should be noted that there was a significant difference between the two groups of primordial prevention and secondary prevention in the expression of BAX gene. Because detraining took 8 weeks in the primordial prevention group, detraining may reduce the positive effects of exercise on breast cancer, so one of the reasons for the significant difference between the two groups could be detraining. On the other hand, the difference between the primary prevention group and the control group in the amount of BCl-2 protein was not significant. Because tumor cell injections were given in the primary prevention group at the same time as exercise, the acute effects of exercise that increase inflammatory factors may have led to a significant decrease in BCl-2 protein levels.

The results of the present study showed that there was no significant difference between the groups in tumor growth rate. This result was consistent with the research of Jones *et al*.[25] and Noorshahi *et al*.[37] and was inconsistent with the Rafiei *et al*.[28] and Agha Ali Nejad and Hashemi[38] researches. One of the possible reasons for the difference in the results of the present study with the research of Rafiei *et al*. can be the length of the training period in the mentioned study. Also, in the research of Rafiei *et al*., the tumor volume index has been compared, while in the present study, the tumor growth rate has been studied. On the other hand, in the study of Agha Ali Nejad and Hashemi, effect of aerobic exercise with selenium nanoparticles on tumor volume was investigated. In the same study, aerobic exercise alone did not significantly reduce tumor size. Short-term training can be one of the possible reasons for the lack of tumor growth in the present study. On the other hand, the primordial and primary groups had 8 and 4 weeks of detraining, respectively, after 4 weeks of aerobic exercise. Detraining, unlike regular exercise, increases tumor growth.[39]

Conclusions

Aerobic exercise reduces the tumor cells' growth through

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different mechanisms, one of which is the available apoptosis in tumor cells. In this study, the effect of 4 weeks aerobic exercise on some apoptotic genes and proteins in cancer-infected mice is assessed. The results indicate that aerobic exercise decreases the antiapoptotic genes and proteins expression and increases the pro-apoptotic genes and proteins expression; thus, no difference is observed in tumor growth. It seems that more effective assessment must be run on the effect of longer aerobic training periods on tumor growth. Appearance of apoptosis in tumor cells is the first phenomenon to control the growth therein; consequently, it can be concluded that aerobic exercise may improve the patient's health by increasing apoptosis in cancer cells; therefore, the breast cancer doctors prescribe aerobic exercise to their patients in addition to conventional treatments for better and faster recovery.

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Conflicts of interest

There are no conflicts of interest.

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