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Research Article

The effect of aerobic exercise training on the expression of genes involved in cardiac apoptosis (Caspase-3/-7) in rats with glioblastoma multiforme

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Abstract

Performing aerobic exercise in different disease conditions can regulate cardiac homeostasis and reduce cardiac apoptosis caused by the disease. In brain cancer, other tissues, including cardiac tissue, can also be affected. Since exercise training causes organ crosstalk, in this study, the effects of aerobic exercise training (AET) on cardiac apoptosis in Glioblastoma multiforme (GBM) rats are evaluated. Twenty-four male Wistar rats were divided into 3 groups (n=8 in each) of healthy control, GBM, and GBM+AET. Glioblastoma was injected into the frontal cortex of rats. The training group (AET) performed aerobic exercises on the treadmill for 4 weeks, 3 days a week at a speed of 18 meters per minute, for 25-40 minutes. In the end, the rats were sacrificed and caspase-3 and caspase-7 were analyzed from the myocardium by Real-time PCR method. Considering H&E image, the GBM group showed necrosis and apoptosis in cardiac tissue compared to the healthy group. Compared to the healthy control group, GBM significantly increased caspase-3 and caspase-7 mRNA in the myocardium ($p<0.05$). However, in contrast to the GBM group, the GBM+AET showed a significant decrease in caspase-3 and caspase-7 mRNA at the myocardium ($p<0.05$). Since tumor formation in the body can affect other distant tissues in an endocrine manner, it is suggested to prioritize aerobic exercise to control the damage caused by GBM on heart tissue. However, more studies are needed, especially on human samples.

Key Words: **Glioblastoma multiforme, Aerobic exercise, Cardiac apoptosis, Caspase-3, Caspase-7**

Introduction

Glioblastoma (GBM) is one of the most lethal and recalcitrant solid malignant tumors (Sharif et al., 2023). In the United States alone, approximately 12,120 patients with GBM were diagnosed in 2016, with a 5-year survival rate and a high mortality rate in these patients. Malignant gliomas, including GBM, are rare. The peak age-adjusted incidence of GBM in the United States is estimated to be 3.2 per 100,000 populations (Ostrom et al., 2015). The incidence of this cancer increases dramatically after the age of 54 and reaches its peak between the ages of 75 and 84. Due to the higher incidence rate in the elderly and the increase in life expectancy in developed countries, the average age of GBM has increased to 64 years in the last few decades.

The immobility caused by the tumor or even the secretions caused by the tumor (oxidative stress and inflammation) causes the tumor tissue to affect distant tissues in an endocrine manner. It has been stated that metastasis is the main cause of death in cancer patients. Inflammatory processes following the interaction between tumor cells and the tumor microenvironment play an important role in cancer progression and metastasis (Hosseini et al., 2017). As tumors develop, tumor cells secrete various chemokines to attract monocytes to infiltrate tumor tissues (Ge & Ding, 2020). Inflammation caused by tumors or inactivity can also affect muscle tissues throughout of body.

The heart muscle can lose its homeostasis under the influence of disease or a sedentary lifestyle. Apoptosis of heart cells can occur due to physiological and pathological reasons caused by disease. Apoptosis can be detected by the caspase pathway. Caspase-3, a cysteine-aspartic acid protease, has recently attracted much attention due to its incredible role in tissue differentiation, regeneration, and neurodevelopment. This enzyme is a key zymogen in cell apoptosis and is not activated until it is cleaved by initiator caspases during the apoptotic flux (Asadi et al., 2022). In addition to caspase 3, it has also been show that efficient apoptosis requires feedback amplification of

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upstream apoptotic signals by caspase 3 or 7 (McComb et al., 2019). Therefore, in this study, caspase activity was evaluated in the heart tissue of healthy and GBM rats to check the amount of apoptosis. Since exercise is one of the most effective ways to control damage caused by disease and a sedentary lifestyle, in this study, aerobic exercise is evaluated as a therapeutic target to control caspase activity in the heart of rats with GBM.

There is controversy as to whether the effects of endurance exercise on the oxidative status and antioxidant defense systems of the myocardium are reduced, increased, or even unchanged (for a comprehensive review see Ji, 1999) (Ji, 1999). Some disagreements may arise from different methods used for determinations and differences in the models employed (running vs. swimming, mice vs. mice, male vs. female). In this regard, it is worth noting that the total volume of exercise (duration and intensity) can be a critical factor in inducing adaptation, as previously suggested by Powers et al. Most studies use moderate-intensity protocols that are more than 12 weeks (Arabzadeh et al., 2022; Chiang et al., 2019). Considering the ability of the myocardium to regulate its antioxidant defense and control the process of apoptosis, the total training volume can be suitable for creating adaptation. On the other hand, no study has shown definitive justification for the functional role of aerobic exercise in the pathological control of apoptosis in cardiac cells in brain cancer. Therefore, in this study, we examine the effect of aerobic exercise training on the expression of genes involved in cardiac apoptosis (caspase-3 and caspase-7) in rats with glioblastoma multiforme.

Materials and Methods

Animals

Twenty-four 8-week-old Wistar rats (223±16.99 grams) were purchased from Pasteur Institute, Tehran, Iran. The rats were placed individually in transparent polycarbonate cages (all 5 rats in one cage) under laboratory conditions of 22±2 degrees Celsius and relative humidity of 55% and a 12-hour light-dark cycle. Standard pellet food and water were freely available to the rats. After one-week of familiarization with the laboratory environment and training on the treadmill, the rats were divided into 3 groups (n=8 in each group), healthy control, GBM, and GBM+AET. The study was approved by the Ethics Committee of the Islamic Azad University, Tehran, Iran under protocol number IR.IAU.SRB.REC.1401.029.

Culture of glioma cells

The C6 glioma cells of the Wistar rats (National Center for Genetic Resources) were prepared in a flask in RPMI medium (Roswell Park Memorial Institute), 300 mg/ml penicillin, 720 mg/ml streptomycin (Jabarban Hayan Pharmaceuticals) and were cultivated 2 g/liter sodium bicarbonate 10%. The final volume of the cell culture medium was 1000 ml; its pH was adjusted to 1.7. After washing, the supernatant was neutralized with PBS (buffered saline Pho) and 0.025% trypsin-EDTA solution and with 10% FBS medium. Then the solution was centrifuged at 1200 rpm for 5 minutes and the cells were separated. The initial density for cell culture was considered to be 100,000 cells/cm2. Finally, 10 microliters of trypan blue dye (0.4% weight-volume) and 90 microliters of cell suspension and neobar slide were used for cell counting and survival. The percentage of stained cells (blue) was determined as the percentage of dead cells.

Injection of glioma cells

To inject cancer cells, animals were first anesthetized using ketamine (80 mg/kg) and xylazine (10 mg/kg). cultured C6 glioma blastoma cells were injected with a concentration of 5*105 cells/30 µL by making a skin incision in the back of the skull and removing the periosteum according to Swanson's instructions using an infusion pump and a stereotaxic device in the right frontal cortex area with a depth of 2.5 mm in rats to a volume of 10 microliters. The tumor size was measured by a digital caliper after sacrificing the animals. Tumor grading was graded from 1 to 4. Grade 4 is the highest degree of damage and grade 1 is the lowest amount of tissue damage (Swanson, 2018).

Table 1. Aerobic exercise training (AET)

	Speed (m/min)	Duration (min)	Frequency (day/week)
Familiarization	5-10	5-10	3
Tumor induction		Injection of glioma cells (1 weeks to confirming)	
Week 1	18	25	3
Week 2	18	30	3
Week 3	18	35	3
Week 4	18	40	3

Table 2. The sequence of used primers for the studied variables.

Genes name	Primer sequences	Accession number
Caspase-3	Forward: GAGCTTGGAACGCGAAGAAA	NM_21578.2
	Reverse: GCCCATTTCAGGGTAATCCA	
Caspase-7	Forward: ACGGTACGCGAAGAAAAGTGAC	NM_022266.2
	Reverse: TCCTGACTTCGTATTTCAGGGC	
GAPDH	Forward: TCCACGATCAAAGCTGTCCT	NM_001107754.2
	Reverse: CGTGCCAAGTGATTCCTCTG	

Aerobic exercise

After confirming the brain tumor, the exercise protocol was designed and started based on the protocol of Al-Jarrah et al. According to this protocol, to reduce stress and adapt to the conditions of the treadmill, the rats walked on the treadmill for one week at a speed of 5-10 m/min for 5-10 minutes and 3 days a week. According to Table 1, exercise groups performed aerobic exercises for 4 weeks, 3 days a week, and at a speed of 18 meters per minute on a treadmill. The duration of training for adaptation in the first week was 25 minutes a day, with a weekly increase of 5 minutes. This duration reached 40 minutes in the fourth week, which continued until the end of the week (Al-Jarrah et al., 2010). For each exercise training session, we consider a 5-minute warm-up and a 5-minute cool-down. The control group was put the near the treadmill during training session.

Gene expression analysis

48 hours after the last training session, Rats were sacrificed after being anesthetized with xylazine and ketamine solution. The heart tissue was immediately frozen in liquid nitrogen and kept at freezer at -70 degrees Celsius. Heart tissue was sent to the laboratory to continue the extraction of RNA (RiboNucleic Acid). caspase-3 and caspase-7 genes were measured after RNA extraction and cDNA (Complementary deoxyribonucleic acid) production using the Real-time PCR method.

RNA extraction and cDNA production

To extract total RNA, it was homogenized at a ratio of 1 to 10 in Isol RNA-reagent Lysis according to the instructions of the kit (Qiagen, Germany). To remove the protein components, the resulting product was centrifuged at 4C for 10 minutes at 12000 rpm. The supernatant was removed and mixed with chloroform with primary Isol at a ratio of 0.5 to 1. The product was centrifuged at 4C for 15 minutes at 12000 rpm. The mineral and aqueous parts were separated, and the RNA-containing part was removed and mixed with isopropanol at a ratio of 0.5 to 1 and left for 10 minutes at room temperature and then at 4o^{C} for 10 minutes. Then it was centrifuged at 12000 rpm. The plate containing RNA was dissolved in 20 µL of Free-RNAs water. The concentration of RNA was measured using a nono drop device and the ratio of 260 to 280 between 1.8 and 2 was defined as optimal purity. After extracting RNA with high purity and concentration from all the studied samples, the stages of cDNA synthesis were performed according to the manufacturer's protocol (Fermentas, USA). Then the synthesized cDNA was used to perform the reverse transcription reaction.

Real-time PCR

Distilled water containing 10 microliters of lyophilized primer, 0.5 microliters of forward primer and reverse primer (Primer Reverse), 1 microliter of cDNA, and 8 microliters of DEPC water was used to prepare the primers. For Biagen, the total RNA of the cells was extracted according to the Cinagen protocol using the q RT-PCR method using Kiazol solution (Cinagene, Tehran, Iran). The quality of extracted RNAs was evaluated by spectrophotometry. To prepare single-stranded cDNA, Oligo dt primer and reverse transcription enzyme were performed according to the relevant protocol. Each PCR reaction was performed in an ABI Step One machine according to the manufacturer's protocol. Real-time PCR reaction cycles for caspase-3 and caspase-7 genes were performed at three temperatures of 94, 60, and 72 degrees Celsius. A melting chart was performed to check the accuracy of PCR reactions. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as caspase-3 and caspase-7 reference genes. The expression levels of control and experimental genes were measured together. The fold change for each gene was determined after normalization to GAPDH using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

$$\Delta CT = CT_{target} - Ct_{reference}$$

$$\Delta\Delta Ct = \Delta CT_{test\ sample} - \Delta CT_{control\ sample}$$

Relative expression: $2^{-\Delta\Delta Ct}$

The results are represented as the mean (±standard error of mean SEM) fold changes concerning the sham control.

Primer sequences used are shown in Table 2.

Brain histology

Brain tissue was removed and fixed in 4% buffered formalin. The formalin-fixed brain was embedded in paraffin, sectioned at 5-µm thickness, and stained with hematoxylin and eosin (H&E). Light microscopy is used for standard histopathological evaluation (FEI Company, Eindhoven, The Netherlands). Histological analysis was evaluated based on the scoring criteria.

Statistical analysis

To determine the normality of the data, we use the Shapiro-Wilk test. Results are expressed as mean and standard deviation. Variables were compared by analysis of variance (ANOVA) and Tukey's test. Statistical significance: $p < 0.05$. Graph pad prism software was used to draw graphs.

Results

Histological change of brain tissue

The histological changes of the brain tissue using the hematoxylin and eosin (H&E) technique in different research groups to confirm GBM are shown in Figure 1. In the control group, the brain tissue is coherent and uniform, and the hematoxylin color is in the nucleus and eosin in the cytoplasm. Cells exist uniformly and the rate of neuronal cell death is very low. The nucleus of living cells is completely round and somewhat transparent. Most of the time the nuclei are visible. Examining the images in different groups showed that the size of the tumor in the GBM group was larger than in the other groups. Also, in this group, many cells were observed in the mitosis phase, which indicates the high activity of cells in the tumor and led to an increase in angiogenesis and blood supply to the tissue. The amount of apoptosis in this group (GBM) was lower compared to other groups, and parts of the tumor had necrosis, which may be due to the lack of oxygen in the center of the tumor. From the examination of the images in the GBM+AET group, it was found that the tumor size decreased compared to the model group. Cell proliferation and angiogenesis were also reduced in the GBM+AET group (Fig. 1).

Cardiac mRNA expression of caspase-3 and 7

In the present study, cardiac caspase activity was investigated to investigate apoptosis. Changes in cardiac caspase-3 and caspase-7 mRNA are shown in figures 2 and 3, respectively. The results of one-way ANOVA showed that there is a significant difference between different groups in the expression of caspase-3 and caspase-7 ($p<0.05$).

The induction of GBM in the brain tissue caused a significant increase in the expression of cardiac caspase-3 and caspase-7 genes compared to the healthy control group ($p<0.0001$). The increased expression of cardiac caspase-3 and caspase-7 genes in the GBM+AET group was also significant compared to the control group ($p<0.05$). Compared to the GBM group, the GBM+AET group also showed a significant decrease in the expression of cardiac caspase-3 and caspase-7 genes (Fig. 2 & 3).

Discussion

Moderate-intensity aerobic exercise training can reduce the risk of cell injury, oxidative stress, and inflammatory signals caused by mechanical and oxidant disorders (Farinha et al., 2015). Exercise training improves cardiovascular capacity and reduces the risk of cardiovascular disease at different ages (Gremeaux et al., 2012). Exercise has the potential to reduce apoptosis through the upregulation of protective stress-sensitive proteins including nuclear factor kappaB, insulin-like growth factor, and heat shock proteins (Morton et al., 2009). However, the mechanisms by which exercise training improves cardiac apoptosis in cancer patients such as brain cancer are not well defined. Therefore, in this study, we examine the effect of aerobic exercise training on the expression of genes involved in cardiac apoptosis (caspase-3 and caspase-7) in rats with glioblastoma multiforme.

Based on the results of the present study, it was found that during cancer (brain cancer induced by glioblastoma), doing aerobic exercise with moderate intensity (3 days a week) was able to increase the values of apoptotic indicators of heart tissue (caspase-3 and caspase-7). The results of the present study are in some ways consistent with the research of Arabzadeh et al. (2022), who investigated the effect of aerobic exercise and green

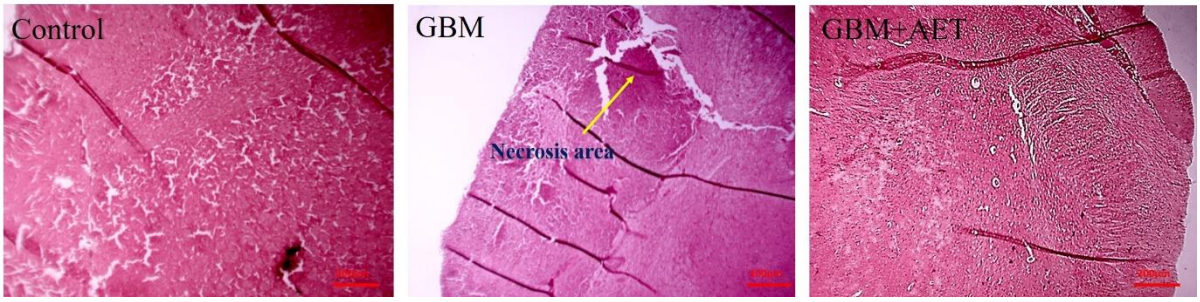

Figure 1. Hematoxylin and eosin (H&E) staining of brain tissue for considering GBM destruction (magnification 200 um). The GBM group shows a wide necrosis in brain tissue. GBM: Glioblastoma Multiforme, AET: Aerobic Exercise.

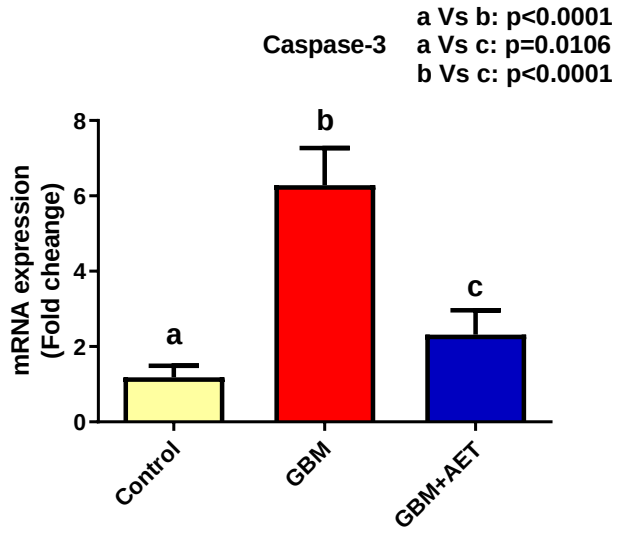

Figure 2. mRNA expression levels of caspase-3 gene in the myocardium of healthy and GBM groups. Values are expressed as mean ± SD (n = 8). Different letters indicate statistically significant differences between groups at p < 0.05. GBM: Glioblastoma Multiforme, AET: Aerobic Exercise.

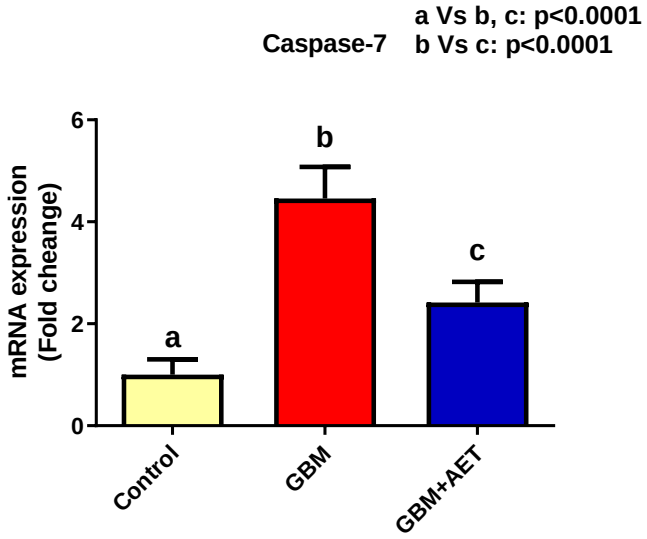

Figure 3. mRNA expression levels caspase-7 gene in the myocardium of healthy and GBM groups. Values are expressed as mean ± SD (n = 8). Different letters indicate statistically significant differences between groups at p < 0.05. GBM: Glioblastoma Multiforme, AET: Aerobic Exercise.

tea supplementation on myocyte apoptosis. These researchers stated that performing aerobic exercise for 12 weeks can significantly reduce cardiac apoptosis indices (Arabzadeh et al., 2022). However, in this study, aerobic training was used for a shorter period of time, i.e. 4 weeks, in the cancer model, and considering that cancer models, especially brain GBM, cannot train for a long time, so this type of aerobic training can also be effective. It can reduce the damage caused by inactivity at the cellular level. Also, Kwal (2013) shows that endurance exercise training reversed the elevation of apoptotic signaling and apoptosis, suggesting that exercise training protects the heart against apoptosis (Kwak, 2013).

Apoptotic signaling via caspase-dependent pathways induces apoptosis in cardiac tissue. The death-inducing signaling complex activates caspase 8, which subsequently activates caspase 3 (Hofmann, 1999). Fourteen caspases have now been identified in mammals that lead to apoptotic cell death (Kumar, 1999). Caspases play a pivotal role in apoptotic pathways and interact with non-caspase apoptotic pathways including endonuclease G (Endo G) and apoptosis-inducing factor (AIF) (Zhang, 2003). It has been shown that exercise training significantly decreases the level of caspase-9 and caspase-3 as well as the ratio of Bax/Bcl-2 in the heart of aged animals (Kwak, 2013). Decreasing this pathway most likely leads to reduced DNA fragmentation. Therefore, cancer or its distant controls or the immobility caused by it increases the apoptotic signals of the Bcl-2 family in the heart, and exercise training in the heart leads to the improvement of the changes caused by the disease in the ap-

-optotic pathways mediated by mitochondria and caspase control (down-regulation of caspase 3 and 7).

Conclusion

The results of this study showed that creating a cancer model in the brain tissue (with GBM), in addition to interfering with the brain itself, can cause cell damage to other tissues, including the heart tissue, and with the spread of apoptosis in the heart, it can cause heart disease and fibrosis. The occurrence of this type of cellular risk factor in the cancer models can increase the death rate. Therefore, doing light aerobic exercise even for a short time can prevent these types of injuries. The results of the present study also confirmed the reduction of two apoptotic indices with 4 weeks of aerobic exercise.

What is already known on this subject?

Performing aerobic exercise in different disease conditions can regulate cardiac homeostasis and reduce cardiac apoptosis caused by the disease.

What this study adds?

Doing light aerobic exercise even for a short period can prevent these types of injuries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Ethics Committee of the Islamic Azad University, Tehran, Iran under protocol number IR.IAU.SRB.REC.1401.029.

Informed consent Animal study.

Author contributions

Conceptualization: N.T, F.Gh.; Methodology: H.Sh, M.Gh.; Software: H.A.; Validation: F.Gh.; Formal analysis: N.T, F.Gh.; Investigation: H.Sh, M.Gh.; Resources: H.Sh.; Data curation: N.T.; Writing - original draft: N.T, F.Gh.; Writing - review & editing: H.Sh, M.Gh.; Visualization: H.A.; Supervision: F.Gh; Project administration: H.Sh.; Funding acquisition: F.Gh.

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