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Research Paper

The EffEct of Combined Ferulic Acid-zinc Oxide and Aerobic Exercise Training on the Prevention of Breast Cancer in Rats

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ABSTRACT

Background: Breast cancer accounts for 23% of all neoplasias in women, which significantly impacts their physical, mental, and social aspects of life. This study investigated the effect of 8-week aerobic exercise alone or with ferulic acid administration combined with or without zinc oxide on the prevention of breast cancer in a rat model.

Methods: A total of 27 rats were randomly divided into 9 groups of 3 rats each as follows: 1) Exercise only; 2) Exercise + ferulic acid; 3) Exercise + zinc; 4) Exercise + ferulic + zinc; 5) Ferulic acid only; 6) Zinc; 7) Ferulic + zinc; 8) Positive controls; and 9) Negative controls. In the first week, the aerobic exercise protocol consisted of a 10-minute warm-up period at a speed of 10 m/min for 20 min, which increased to a speed of 18 m/min for 30 min. The ferulic acid supplement was administered to rats intraperitoneally using insulin syringes with a volume of 200 µL. After the last training session, the rats' breast tissue samples were excised, and the expression levels of BCL2, Bax, and caspase-3 were measured by the real-time polymerase chain reaction method. The data were analyzed by one-way ANOVA and Tukey's post hoc test ($P \geq 0.05$).

Results: The findings showed that intermittent exercise plus ferulic acid-zinc supplementation decreased the BCL2 level in the breast cancer group ($P=0.004$). Intermittent training supplemented with ferulic acid-zinc increased Bax and caspase-3 levels in the breast cancer group ($P=0.001$).

Conclusion: Aerobic exercise combined with ferulic acid and zinc nanoparticles inhibited cell apoptosis promoted by breast cancer in rats.

Keywords: Breast cancer, Apoptosis, Aerobic exercise, Ferulic acid

Introduction

The increasing incidence of cancer in recent years, along with its effects on numerous physical, mental, and social aspects of human life, has established it as a core issue of the current century [1]. Among different types of neoplasia, breast cancer is responsible for 23% of all cancers in women. A recent systematic review indicates that the common causes of breast, colon, and ovarian cancers are gene mutations, tobacco, processed foods rich in trans fats and sugar, and the lack of exercise [2]. Another factor is the release of estrogen from fat tissue. These factors inhibit programmed cell death (i.e., apoptosis) and an increase in cell proliferation, playing important roles in carcinogenesis [3]. Cell apoptosis is the main mechanism in the development and homeostasis of mature tissues in order to remove unnecessary, infected, mutated, or damaged cells generated due to various physiological and pathological alterations [4].

Bax is a protein that inhibits apoptosis by activating the BCL-2 oncogene. Bax is a very strong inducer of cell death, while the function of BCL-2 is to increase cell survival [5]. Bax induces the permeability of the mitochondrial membrane and the release of cytochrome C from the mitochondria [6]. BCL-2 has the opposite function of Bax, which is apoptosis inhibition and cell preservation. Activation of Bax expression prevents the function of BCL-2 [7]. The ratio of these two proteins influences whether apoptosis occurs, as a higher Bax/BCL-2 ratio is regarded as a reliable indicator of cell apoptosis [8]. The BCL-2 protein is crucial in the genetic processes governing the growth and survival of eukaryotic cells by preventing cell death; consequently, BCL-2 suppressors could serve as a treatment for cancer by diminishing the inhibitory impact of BCL-2 on apoptosis [9].

Caspase-3 is one of the most important proteases involved in the known pathway of apoptosis [10].

Deficiency and down-regulation of caspase-3 are linked to breast carcinogenesis, suggesting that caspase-3 may serve as a biomarker for cancer prevention and treatment. In this respect, research has demonstrated that functional caspase-3 is crucial for the efficacy of radiotherapy and chemotherapy [11].

Consistent physical activity during both adolescence and adulthood can contribute to a decreased risk of breast cancer. Additionally, for survivors of breast cancer, engaging in regular exercise can alleviate the side effects associated with treatments, such as radiotherapy and chemotherapy, while also enhancing survival probabilities [12].

It has been reported that phenolic compounds have important biological properties, such as anti-cancer, anti-toxic, antioxidant, and anti-inflammatory activities [13]. Ferulic acid (FA), a free-radical scavenger, and paracoumaric acid (p-CoA), a member of the hydroxycinnamic acid family, are the primary phenolic compounds found in cereal bran, a significant source of fiber in a nutritious diet. Given that research has indicated the antitumor properties of plant extracts, these extracts are regarded as a practical approach to decreasing the incidence of breast cancer [14]. It has been shown that FA is a strong antioxidant that prevents cell damage in the body. It can help considerably in resolving cardiovascular disease, diabetes, and other inflammatory conditions [15].

Moreover, zinc oxide (ZnO) nanoparticles are a new type of widely used mineral agent, which have attracted the attention of researchers due to their useful physical and chemical properties. These particles have a higher absorption power than ordinary zinc-containing compounds [16]. The beneficial properties of ZnO nanoparticles encompass low dielectric constant, elevated catalytic activity, high chemical stability, the ability to absorb infrared and ultraviolet light, and, most notably, their antibacterial properties. The advancement of the methodology for cancer treatment can be significantly accelerated if the therapeutic and antitumor effects of these compounds are validated [17].

Aim of the Study: Given the rising prevalence of breast cancer and its detrimental effects on human health, there has been an insufficient number of studies examining the effect of aerobic exercise in the presence of FA, with or without ZnO nanoparticles, on the apoptosis of the breast cancer cells in rats. The purpose of this study was to investigate the effect of FA, with or without ZnO, and aerobic exercise on the prevention of breast cancer in rats.

Materials and Methods

Study Design: We used 27 female rats (Balb/C), each weighing approximately 25 g. They were kept in the laboratory at 22±3°C and humidity of 40-50% under 12-hour light-dark cycles (from 6 a.m. to 6 p.m.). The animals had free access to standard food supplied by the Pasteur Institute, Isfahan, Iran. The potable water for the animals was provided in 500 ml bottles.

Ethical Guidelines: The rats were allowed to move freely within their cages and had unrestricted access to food and water *ad libitum*. This research adhered completely to institutional ethical guidelines and the principles of the Helsinki Declaration concerning the use and treatment of experimental animals. All procedures in the study were designed to minimize pain and suffering for the rats. The study protocol received approval from the Research Ethics Committee, Islamic Azad University, Khorasgan branch, Isfahan, Iran (*Ethics Code #:* IR.IAU.KHUISF.REC.1400.293).

Animal Grouping: The rats were randomly divided into nine groups of 6 each and were assigned to the following treatments:

- ***Group 1*** (negative control): Healthy rats received dimethyl sulfoxide (DMSO) diluted in phosphate-buffered saline (PBS) by intraperitoneal injection.
- ***Group 2*** (positive control): Rats with breast cancer received DMSO diluted in PBS by intraperitoneal injection.
- ***Group 3:*** Cancer rats received FA (200 µL) by intraperitoneal injection.
- ***Group 4:*** Cancer rats received ZnO by intraperitoneal injection.
- ***Group 5:*** Cancer rats received FA combined with ZnO (ZnO-FA) intraperitoneally.
- ***Group 6:*** Cancer rats were given physical exercises only.
- ***Group 7:*** Cancer rats received FA by intraperitoneal injection along with physical exercises.
- ***Group 8:*** Cancer rats received ZnO by intraperitoneal injection along with physical exercises.
- ***Group 9:*** Cancer rats received FA bound to ZnO by intraperitoneal injection along with physical exercises.

Induction of Cancer: A carcinogenic cell line (4T1) was used to induce metastatic breast cancer in the rats. These cells were cultured in a laboratory environment in order to achieve the required amount of cells. After counting the cells and ensuring that the cells reached the required number, a cell suspension was prepared at a concentration of 7-10 cells per one mL PBS solution. Finally, a bolus of 106 cells was injected subcutaneously into the top of the right leg of each rat [18].

FA-ZnO Nanoparticles Treatment: Zinc oxide was synthesized by zinc nitrate precursor in the presence of sodium hydroxide through a co-precipitation method. The ZnO was then bound to FA molecules covalently. The complementary morphology of the synthesized ZnO and FA bound to the zinc element was examined by surface electron microscopy. The ZnO, FA, and FA bound to zinc elements were observed by FT-IR optical spectroscopy. The quantity of ZnO in the nanoparticles was determined by atomic absorption spectrometry test.

The supplements were injected intraperitoneally, using insulin syringes at a 45-degree angle and a volume of 200 µL [19].

Exercise Protocol: Breast cancer was induced in the rats, and then aerobic exercise and specific treatments were administered in each animal group. The main aerobic exercise program for the rats in the exercise group was carried out on a treadmill for 8 weeks. The first week of the training program was started at a speed of 10 m/min for 20 min. At the end of the eighth week, the treadmill speed had reached 18 m/min for 30 min [8] (Table 1).

Dissection & Sampling: To anesthetize the rats, a solution containing 50 mg/ml of ketamine and 20 mg/ml of xylazine was administered 48 h following the final training session and 12 h of fasting. Initially, laboratory specialists conducted pain assessments to confirm anesthesia. Once complete anesthesia was verified, the skull of each rat was opened, and the excised brain was promptly stored in nitrogen. For the analysis of Bax and BCL-2 variables, the breast tissue samples were preserved at -80°C.

RNA levels for Bax, BCL-2 & Caspas-3: Following RNA extraction from all breast samples at high purity, cDNA synthesis for each sample was conducted in accordance with the supplier's protocol (Fermentas, USA). The cDNA extracted from the samples was subsequently utilized for the reverse transcription process. To quantify the RNA levels of Bax and BCL-2, a real-time polymerase chain reaction method was employed, using glycer-aldehyde-3-phosphate dehydrogenase as the control gene. The RNA levels for these genes were determined based on the 2-ΔΔCT formula [20]. The primers were developed by Macrogen Company (Rockville, MD, USA), utilizing genetic information obtained from the NCBI GenBank database (NIH, Bethesda, MD, USA). The oligonucleotide sequences for the Bax, BCL-2, and Caspase-3 genes are provided in Table 2.

Statistical Analyses: After data collection, Shapiro-Wilk's test was conducted to assess the normal distribution of the data. Ultimately, the statistical analysis was carried out using a one-way analysis of variance, followed by Tukey's post hoc comparisons among the groups. All statistical evaluations were executed with SPSS software at the P≤0.05 significance level.

Table 1: Schedule for rat exercise on a running wheel.

Week	Running Speed (m/min)	Exercise Duration (min)	Number of sessions per week (days)
1	10	20	5
2	14	25	5
3	14	25	5
4	16	30	5
5	16	30	5
6	18	30	5
7	18	30	5
8	18	30	5

Table 2: Primer sequences used in this study.

Gene	Primer Sequences
Bax	**Forward:** 5'-CCAAGAAGCTGAGCGAGTGT-3' **Reverse:** 5'-CCCAGTTGAAGTTGCCGTCT-3'
Bcl-2	**Forward:** 5'-TCTTTGAGTTCGGTGGGGTC-3' **Reverse:** 5'-GTTCCACAAAGGCATCCCAG-3'
CASP3	**Forward:** 5'-TGAGCCATGGTGAAGAAGGA-3' **Reverse:** 5'-TCGGCCTCCACTGGTATTTT-3'

Results

Changes in mRNA Expression: Bax gene: Changes in the expression of mRNA genes for the apoptosis factors Bax and BCL-2 are shown in Figures 1 and 2. The results of ANOVA analysis for Bax expression in the breast tissue showed a significant increase after 8 weeks of aerobic exercise and FA-ZnO consumption (*P*=0.001, F=6.284). Based on the results of Tukey's follow-up test, Bax in the FA + ZnO group had a significant increase as compared to the positive control group (*P*=0.01). Moreover, FA group (*P*=0.02), FA + ZnO (*P*=0.001), exercise (*P*=0.001), exercise and FA (*P*=0.01), exercise and ZnO (*P*=0.01), and exercise + FA + ZnO (*P*=0.01) increased in the negative control (Figure 1).

The results of the ANOVA test for the expression of BCL-2 in the breast tissue after 8 weeks of aerobic training plus FA-ZnO consumption showed a significant increase (*P*=0.001, F=7.027). Based on the results of Tukey's BCL-2 post hoc test, FA group (*P*=0.02), ZnO group (*P*=0.02), FA + ZnO (*P*=0.001), exercise (*P*=0.001), exercise and FA (*P*=0.01), exercise and ZnO (*P*=0.01), and exercise + FA + ZnO (*P*=0.01) had a significant decrease compared to the negative control group (Figure 2).

The results of ANOVA analysis associated with Caspase-3 expression in the breast tissue showed a significant increase after 8 weeks of aerobic exercise plus FA-ZnO consumption (*P*=0.001, F=22.89). Based on the results of Tukey's follow-up test, Caspase-3 in the FA + ZnO group had a significant increase as compared to the positive control group (*P*=0.01). Finally, FA group (*P*=0.02), FA + ZnO (*P*=0.001), exercise (*P*=0.001), exercise and FA (*P*=0.01), exercise and ZnO (*P*=0.01), and exercise + FA + ZnO (*P*=0.01) increased in negative control (Figure 1)

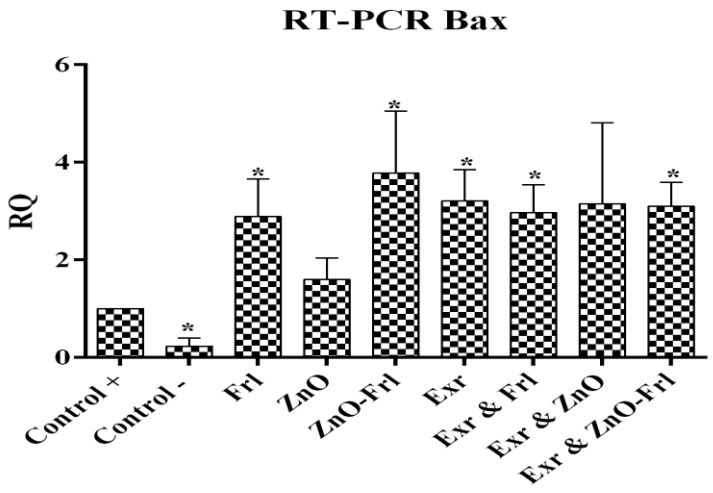

Figure 1: Changes in the expression of mRNA gene for the inflammatory factor Bax across the study group.

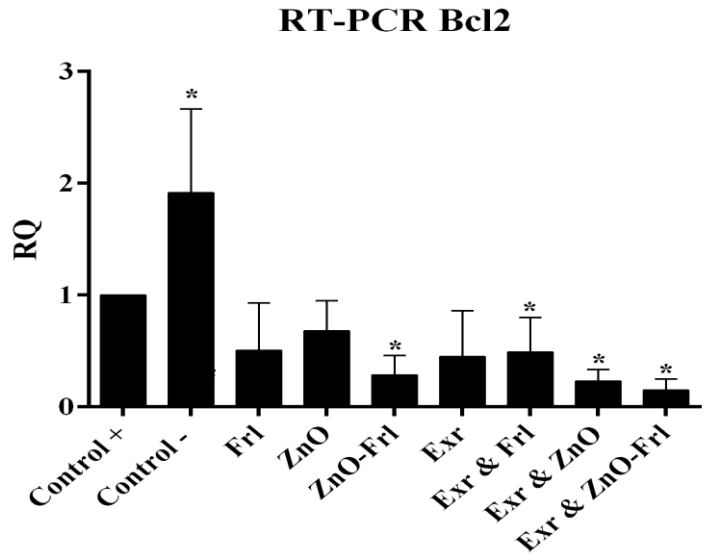

Figure 2: Changes in the expression of mRNA gene for the inflammatory factor BCL-2 across the study groups.

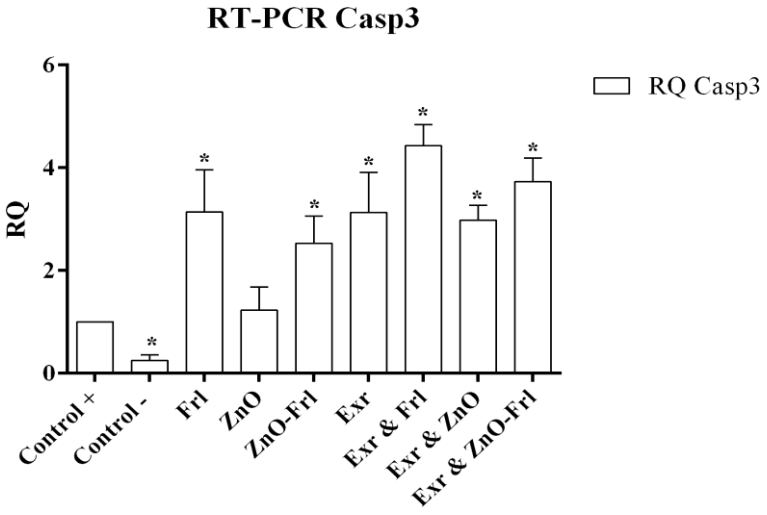

Figure 3: Changes in the expression of mRNA gene for the inflammatory factor caspase-3 across the study groups.

Discussion

Khori *et al*. showed that 5 weeks of exercise alone or in combination with tamoxifen is associated with a decrease in BCL-2 expression, and the decrease in BCL-2 was significant in the tamoxifen, exercise, and exercise + tamoxifen groups compared to the control group [21]. In the present study, exercise plus FA-ZnO was associated with a decline in the BCL-2 expression in the breast tissue. Furthermore, Delfan *et al*. investigated the intratumoral effect of continuous endurance training and intermittent high-intensity training on the expression of miR-21 and BCL-2 genes in female rats with breast cancer. The results showed that there was a significant difference in the BCL-2 gene expression for the two experimental groups compared to that of the control group [22].

It appears that BCL-2 is an effective factor in the prognosis of breast cancer. The expression of BCL-2 has been found in over half of the breast cancers [23]. Some studies have shown that BCL-2 decreases in invasive breast cancer compared to normal tissue and pre-invasive breast lesions. Thus, BCL-2, as an inhibitor of programmed cell death, should be associated with highly invasive tumors and resistant to hormonal cytotoxic therapy [24]. The BCL-2 expression has been demonstrated in more than half of the breast cancers.

Furthermore, BCL-2 is one of the most well-known proteins that inhibit apoptosis, as it not only prevents the release of cytochrome C from mitochondria but also maintains the integrity of the mitochondrial membranes. BCL-2 can also decrease Bax levels in mitochondria by inhibiting its transfer from the cytosol to the mitochondria [25].

It has also been found that the mRNA expression in BCL-2 decreases in cells treated with anti-miR-21. Consequently, miR-21 may regulate BCL-2 expression indirectly, indicating that the inhibitory effects of anti-miR-21 could result in the negative regulation of BCL-2 expression [26]. It is integral to the genetic program governing the growth and survival of eukaryotic cells by preventing cell death. Hence, BCL-2 suppressors could serve as a therapeutic approach for cancer by diminishing its inhibitory influence on apoptosis. In contrast, when Bax is over-expressed, and its homodimers dominate, the cells are subjected to apoptosis [27].

Doxorubicin, an antitumor drug, enhances the Bax effects following the induction of intrinsic apoptosis in breast cancer cells. Some studies have shown that Bax in cells decreases significantly after treatment with doxorubicin [28]. De Lima *et al*. investigated tumor growth with the participation of Bax and Bcl2 following anaerobic exercise. Their results have demonstrated that trained tumor-bearing rats showed tumor cell apoptosis and reduced tumor weight and cell proliferation in vitro compared to those of sedentary rats [29]. Shah Wali *et al*. investigated the effect of aerobic exercise on tumor growth, gene expression, and apoptosis in female rats with breast cancer. The results showed that exercise could probably lead to cell apoptosis in tumors by reducing anti-apoptotic factors [30].

Estrogen inhibits apoptosis through BCL-2 stimulation. At present, there is increasing evidence suggesting the modulatory effect of physical activity on estrogen-induced carcinogenesis in breast cancer through various biologically plausible mechanisms. Thus, exercise can increase apoptosis by decreasing estrogen synthesis, reducing BCL-2, and increasing Bax in breast cancer [31]. The findings of the current study demonstrated that the combined FA and ZnO supplementation plus exercise training reduced BCL-2 and boosted Bax. Several studies have shown that the activation of caspase-3 is necessary for the induction of apoptosis in response to chemotherapy drugs, such as taxane and doxorubicin [32]. Since caspase-3 is a key regulator of apoptosis rate in breast cancer, it may be considered an important marker to predict the response or resistance to chemotherapy drugs.

The change in the Bax/BCL-2 ratio results in the release of cytochrome C from the mitochondria into the cytosol. Subsequently, the cytosolic cytochrome C associates with Apaf-1, which leads to the activation of caspase-3 and PARP. These events highlight the important role of caspases in promoting apoptosis [33]. In another study on human breast cancer, it was observed that about 75% of breast tumor samples lacked caspase-3 transcription and expression. At the same time, the remaining samples also showed a significant decrease in the caspase-3 expression [34]. The results of research by Kazemi *et al*. have shown that 6 weeks of aerobic exercise training has impacted the levels of caspases-3 and -9, resulting in a significant increase in these caspases compared to the control group [35].

Ferulic acid is a phytochemical present in plant cell walls. Phytochemicals with antioxidant properties are generally beneficial to human health. These compounds are known for their anti-cancer, anti-mutation, anti-inflammatory, antioxidant, and anti-toxin properties. Ferulic acid, a phenolic compound found in various cereals, has an inhibitory effect on the growth of breast cancer cells [36]. Studies have shown that this compound has anti-toxic, antacid, anti-apoptotic, and differentiation effects in cancer cells. These findings indicate that the anti-toxic effect of exercise, together with FA and ZnO supplementation, can provide a strategy to control tumor volume and protect against breast cancer. The results of the present study provide evidence that 8-week aerobic exercise plus FA-ZnO supplementation increases apoptosis in breast cancer [37]. Our study findings also show that the simultaneous use of FA-ZnO and exercise reduce the toxic and carcinogenic effects in the rat model of breast cancer.

Conclusions

According to the results of the present study, an eight-week aerobic exercise combined with the consumption of FA-ZnO nanoparticles can decrease BCL-2 and increase Bax and caspase-3. The use of FA-ZnO compound concurrently with aerobic exercise reduces the toxicity of and inflammatory response of breast cancer in the animal model utilized in the current study.

Limitations of the Study: The present study did not involve the examination of changes in other mediators of apoptosis. Nevertheless, it is conceivable that the release and/or activation of additional toxic, inflammatory, and apoptotic factors may have affected the results of the study.

Recommendations for Future Studies: The levels of other apoptotic factors, including P53 and caspase-9, should be measured in order to reach a better insight into the effect of aerobic exercise training and FA-ZnO supplementation on cell apoptosis in patients with breast cancers.

Conflict of Interests

The authors declare that they have no conflict of interests with any internal or external entities in conducting this research project.

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Compliance with Ethical Guidelines

The study protocol was approved by the Research Ethics Committee, Islamic Azad University, Khorasgan Branch, Isfahan, Iran (Ethics Code #: IR.IAU.KHUISF.REC. 1399.046).

Authors' Contributions

Conceptualization and supervision were done by Maryam Pira and Khosro Jalali Dehkordi. The methodology was done by Maryam Pira. Investigation, writing the original and several drafts of the manuscript, review, editing, and approval of the final article were done by all authors.

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