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Effects of exercise training and supplementation with selenium nanoparticle on T-helper 1 and 2 and cytokine levels in tumor tissue of mice bearing the 4 T1 mammary carcinoma

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ABSTRACT

Objectives: Physical exercise decreases the incidence of breast cancer and also improves survival in breast cancer patients. However, the mechanistic basis of these protective effects of exercise is not well known. Changes in tumor cytokines, such as oncostatin-M (OSM), have been associated with modulation of antitumor immune responses in breast cancer. Exercise and antioxidants such as selenium affect both antitumor immune responses as well as tumor cytokine expression. Thus, the aim of this study was to determine the effects of aerobic exercise training (AET) and selenium nanoparticle (SeNP) administration on T-helper 1 and 2 and tumor tissue cytokines in mice bearing the 4 T1 mammary carcinoma.

Methods: We examined the effects of 6 wk of AET and SeNP administration (100 μg three times/wk) on tumor size, concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, IL-4 and OSM in tumor tissue and INF-γ and IL-4 in splenocytes of 64 mice bearing the 4 T1 mammary carcinoma.

Results: AET increased OSM levels in tumor tissue. Moreover, AET increased levels of TNF-α in tumor tissue, whereas SeNP supplementation decreased IL-4 levels tumor tissue. Also, the combination of AET and SeNP administration decreased tumor volume and increased T-helper 1 cytokines in the splenocytes of tumor-bearing mice.

Conclusion: These findings suggest that the combination of AET and SeNP supplementation effects antitumor immune responses in splenocytes, whereas AET induced antitumor cytokines, such as OSM and TNF-α in tumor tissue.

Introduction

Breast cancer is the most common type of cancer in women, and its frequency is increasing in low- and middle-income countries [1,2]. Approximately one in eight women worldwide will develop breast cancer during her lifetime and it is the leading cause of cancer deaths among women [3]. According to the results of some studies, environmental and lifestyle factors, such as diet and physical activity, influence the prevalence and progression of breast cancer [4,5].

Observational research suggests that exercise training may reduce the risk for breast cancer and also may improve survival [6]. Several mechanisms for this protective effect of exercise training have been proposed, including alterations in endogenous hormones, energy balance, DNA repair capacity, and systemic low-grade inflammation [7,8]. However, one proposed mechanism for the protective effect of aerobic exercise related to cancer risk and

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outcomes, but which has not been examined definitively, is the effect of exercise on antitumor immune responses [9].

T-helper (Th) cells play a key role in controlling the immune response [10]. T-helper 1 (Th1)-like cells are principally involved in promoting cell-mediated immunity, initiating a cytotoxic response, and generally are considered to be the main host anticancer mechanism [11,12]. However, in tumor-bearing hosts, because of mechanisms that are employed by growing cancer, immune responses often are not effective [13]. It has been demonstrated that growing cancers actively suppress Th1 immune responses [14,15].

It has been proposed that cytokines, which modulate immune cells, are responsive to exercise training, and their balance in the tumor microenvironment may reflect immune phenotype in the tumor [9]. Regular exercise training is associated with enhanced immune responses, particularly through promotion of cell-mediated immune responses [16–18]. In this regard, some cytokines that are released from muscle tissue after exercise, termed *myokines*, may be an important modulator of host antitumor immune responses [19,20]. Myokine oncostatin-M (OSM) has been shown to inhibit mammary cell proliferation by inducing apoptosis in these cells [20].

In addition, recent evidence suggests that higher intensity levels of exercise are effective in decreasing the risk for breast cancer mortality [21,22]. Velez et al. showed that training intensity is a predictor of the effects of exercise training on tumor markers [23]. It is known that high-intensity aerobic exercise is associated with increases in stress factors that have suppressive effects on the immune system [24,25]. Dietary antioxidant supplementation can afford protection against exercise-induced stress [26]. Selenium (Se) is an important micronutrient ion that has broad effects on biological systems, including antioxidant and anticarcinogenic effects as well as cancer prevention [27,28]. Se nanoparticles (SeNPs) are attracting increasing attention due to their excellent biological activities and low toxicity. Also, in animal studies, SeNPs have been shown to have antitumor activity and could increase immune responses [29,30].

The tumor microenvironment creates a feedback loop of proinflammatory signaling via the upregulation and production of specific cytokines [31]. Because tumor cytokines, such as interferon (INF)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-4, play a key role in the tumor progression in breast cancer, aerobic interval training might represent a simple strategy for modulating expression of these cytokines and hence affect both tumor progression and antitumor immune responses. Also, the immunologic effects of SeNPs and exercise training, especially in Th1 immune responses, were observed in some other studies [30,32]. Thus, the present study tested the hypothesis that SeNPs and aerobic exercise training (AET) modulate tumor cytokines and increase Th1 cytokine production in spleen cell culture in mice bearing the 4 T1 mammary carcinoma cell line.

Methods and materials

Animals and experimental design

Experimental protocols using mice were conducted following the policies of the Iranian Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, and authorized by the Ethics Committee of the School of Medical Sciences, Tarbiat Modares University, Tehran, Iran. Inbred female BALB/c mice (aged 6–8 wk, obtained from Iran Pasteur Institute) were housed in a temperature-controlled room with alternating 12-h light and dark periods and were fed via a standard mouse pellet diet. Animals were maintained in the Central Animal House, School of Medical Sciences of Tarbiat Modares University.

At start and before tumor injection, animals (16 in each group) were randomly distributed to the following groups: control (C); trained (E); selenium nanoparticles (Se); and selenium nanoparticles plus training (SeE). The four groups were subjected to oral SeNP supplementation and AET for 6 wk. After 6 wk of treatment, animals in each group were divided in two subgroups (n = 8 per group): Tumor-bearing control (CT); control normal (C); tumor-bearing trained (ET); normal trained (E), tumor-bearing SeNP (SeT); normal SeNP (Se); tumor-bearing SeNP plus training (SeET); normal SeNP plus training (SeE). SeNP supplementation and AET continued for another 6 wk. SeNP groups were supplemented with 100 μg of SeNPs three times per week. All treatments were administrated by oral gavage. Untreated or control mice were administrated using oral gavage method by needle only.

Preparation of SeNPs

SeNPs were prepared with a method previously described [33,34]. A solution of 5.2 mM selenium dioxide (Merck, Germany) was prepared and aqueous ascorbic acid solution (5.2 mM) was added into the mixture with continuous stirring (300*g*) with a magnetic stirrer. The resulting reaction mixture was centrifuged and washed three times with double-distilled water. A stock solution of SeNPs (1 mg/mL) was prepared and used for further oral administration in doses of 100 and 200 μL per mouse.

Tumorgenicity

We trypsinized and resuspended 4 T1 cells (estrogen receptor [ER]/progesterone receptor [PR]-negative, National Cell Bank of Iran, Pasteur Institute, Tehran, Iran) in 10-fold excess culture medium. After centrifugation, cells were resuspended in phosphate-buffered solution, and 1×10^6 cells were injected (0.1 mL, subcutaneous) using a 21-gauge needle into the left flank of BALB/c mice under ketamine and xylazine (10 mg/kg, intraperitoneal) anesthesia. Visible tumors were observed about 2 wk after cancer induction.

Measurement of tumor volume

Tumors were sized in two dimensions. The larger tumor dimension was considered as length (L), and the other (at 90 degrees) as width (W). After appearance of the tumor, the length and width of the tumor were sized by a digital caliper weekly. Tumor volume was then accounted with the following tumor volume formula: $[V = \pi/6\,(W \times L^2)]$.

AET protocol and aerobic exercise test

Training groups performed aerobic interval training on a treadmill for 5 d/wk, for 6 wk before tumor injection and 6 wk afterward. The aerobic interval training protocol was based on performance of pilot studies and according to previous literature reports [35–37]. Mice in the training groups performed an aerobic interval training protocol comprised of a 10-min warm-up, and ten 2 min intervals of running at 70% V_{max} separated by 2 min of active recovery at 50% V_{max}. Maximal exercise capacity was tested every 2 wk for determination of peak velocity in a subset of normal and tumor-bearing mice. Following a 10-min warm-up, the maximal exercise test was started from a speed of 6 m/min. Treadmill speeds were increased 3 m/min, every 3 min until exhaustion, which was defined as the point at which animals could not maintain the running speed despite a gentle tap on the tail more than five times [38,39].

Tissue preparation

Twenty-four hours after the last training session, mice were anesthetized with a mixture of ketamine and xylazine (10 mg/kg bodyweight, intraperitoneal). Tumor tissue was quickly extracted, and stored in liquid nitrogen for subsequent analysis.

Tumor preparation

After elimination of the central necrotic part of tumor, whole tumor tissue was homogenized in ice-cold radioimmunoprecipitation buffer (0.625% Nonidet *P*-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, 1 mM Ethylenediaminetetraacetic acid (EDTA) at pH 7.4, plus 10 μg/mL of a protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]). Homogenates were centrifuged at 12 000*g* for 10 min at 4°C, and the supernatant was saved. Protein content was measured via the Bradford assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a reference [40]. Supernatants were stored at −80°C.

Assay of cytokines in tumor

TNF-α, IL-6, IL-4, and INF-γ protein levels in tumor tissue were determined in duplicate using commercially available mice enzyme-linked immunosorbent assay (ELISA) kits (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA). OSM protein concentrations in tumor were determined in duplicate using the CUSABIO ELISA

kit (Cusabio Life Science Inc., Washington, DC, USA). The assays were carried out according to the manufacturer's instructions. The minimum detectable concentrations were <10.9 pg/mL for TNF-α, <1.6 pg/mL for IL-6, <2 pg/mL for IL-4 and INF-γ, and <3.12 pg/mL for OSM. The intra- and interassay coefficients of variation were 3.9% and 6.2% for TNF-α, 4.7% and 5.2% for IL-4, 4.7% and 7.53% for IL-6, 3.6% and 9.33% for INF-γ, and 8% and 10% for OSM.

Cytokine determination in spleen cell cultures

At time of sacrifice, spleens of experimental mice were separated, dissected mechanically, and suspended in cold phosphate-buffered solution containing 2% of fetal bovine serum. In the next, red blood cells were lysed using mouse red blood cell lysis buffer and the suspension was centrifuged at 300*g* for 10 min at 4°C. The cell pellet was resuspended in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 IU/mL penicillin, and 100 μg/mL streptomycin. A total number of 3×10^6 spleen cells was seeded into each well of 24-well plate in complete RPMI-1640 and antigen recall carried out in vitro with adding three multiplicity of infection-inactivated acyclovir-susceptible strain (KOS). The plates were incubated at 37°C in 5% carbon dioxide. Three days after antigen recall, supernatants were removed and the concentrations of IFN-γ and IL-4, as indexes of Th1 and Th2 cell activation, were estimated by quantitative ELISA kit (R&D Systems) according to the manufacturer's instruction. The concentration of each sample (pg/mL) was calculated according to the standard curve.

Statistical analysis

All analyses were performed using SPSS version 16 (SPSS, Chicago, IL, USA). Three-way analysis of variance (ANOVA) and Tukey post hoc tests were used for SeNPs, cancer, and AET effects. Also, two-way ANOVA and Tukey post hoc tests were used for tumor data. Statistical significance was set at $P < 0.05$. Data are presented as means ± SEM.

Results

Tumorigenesis

Tumor volumes were measured via external palpation weekly and were reported elsewhere [41]. Except for weeks 2 and 5, tumor volume showed a significant effect of SeNP supplementation (Fig. 1). Also, in the sixth week after tumor injection, a significant effect of AET on tumor volume was observed. The SeET group, which underwent both aerobic interval training and SeNP administration, exhibited lower tumor volume than other tumor-bearing groups.

Cytokine levels in tumor tissue

Figure 2 shows the effects of SeNAs and AET on protein levels of cytokines in tumor tissue. Aerobic interval training increased TNF-α in tumor tissue ($P < 0.05$; Fig. 2A), whereas aerobic interval training plus SeNAs had no effects on INF-γ and IL-6 protein levels in tumor tissue (Fig. 2B, C). A significant effect of SeNP administration and a training × SeNA effect was observed for IL-4 levels in tumor tissue (Fig 2D). Post hoc analysis indicated that IL-4 protein levels were higher in the CT group than in the three other tumor-bearing groups ($P < 0.05$). In addition, significant effects of training were observed for levels of OSM in tumor tissue ($P < 0.05$; Fig. 2E). These findings indicated that AET induced antitumor cytokines such as TNF-α and OSM in tumor tissue.

Stimulated cultures from trained non–tumor-bearing mice that received SeNPs showed significant increases in INF-γ levels (Fig. 3A). However, in tumor-bearing mice, AET and SeNPs significantly increased INF-γ levels (cancer × training × SeNP interaction; $P < 0.05$). Post hoc analysis indicated that INF-γ concentrations were lower in the CT group than in the three other tumor-bearing groups ($P < 0.05$). Also, IL-4 concentrations, an index of Th2 cell activation, increased in tumor-bearing mice (Fig. 3B). AET and SeNPs significantly decreased IL-4 concentration (cancer × training × SeNP interaction; $P < 0.05$). Post hoc analysis demonstrated that the CT group had higher levels of IL-4 in splenocytes than tumor-bearing mice in other groups ($P < 0.05$). As shown in Figure 3C, the ratio of INF-γ to IL-4 increased after AET and SeNP supplementation ($P < 0.05$). These results indicate that aerobic interval training and SeNP administration, alone or in combination, could boost the antitumor Th1 cytokine profile and inhibit the Th2 phenotype.

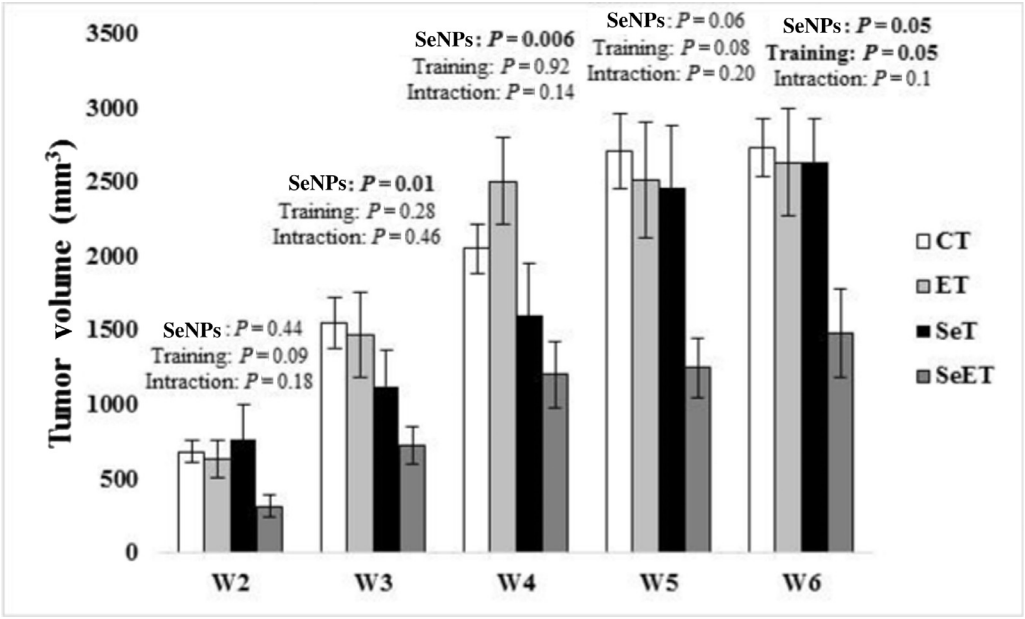

Fig. 1. Effects of aerobic interval training and selenium nanoparticles supplementation on tumor volume. Tumor volume determined weekly (W2–W6). In all weeks after tumor injection except the first measurement, selenium nanoparticle supplementation significantly reduced tumor volume. At 6 wk after tumor injection, a significant effect of training was observed in tumor volume. Groups for tumor-bearing mice are CT, control sedentary; ET, trained; SeT, selenium nanoparticles administrators; SeET, selenium nanoparticles administrators plus training. Results are expressed as mean ± SE. Results from the two-way analysis of variance are presented. N = 6 to 8 animals per group [39]. SE, standard error.

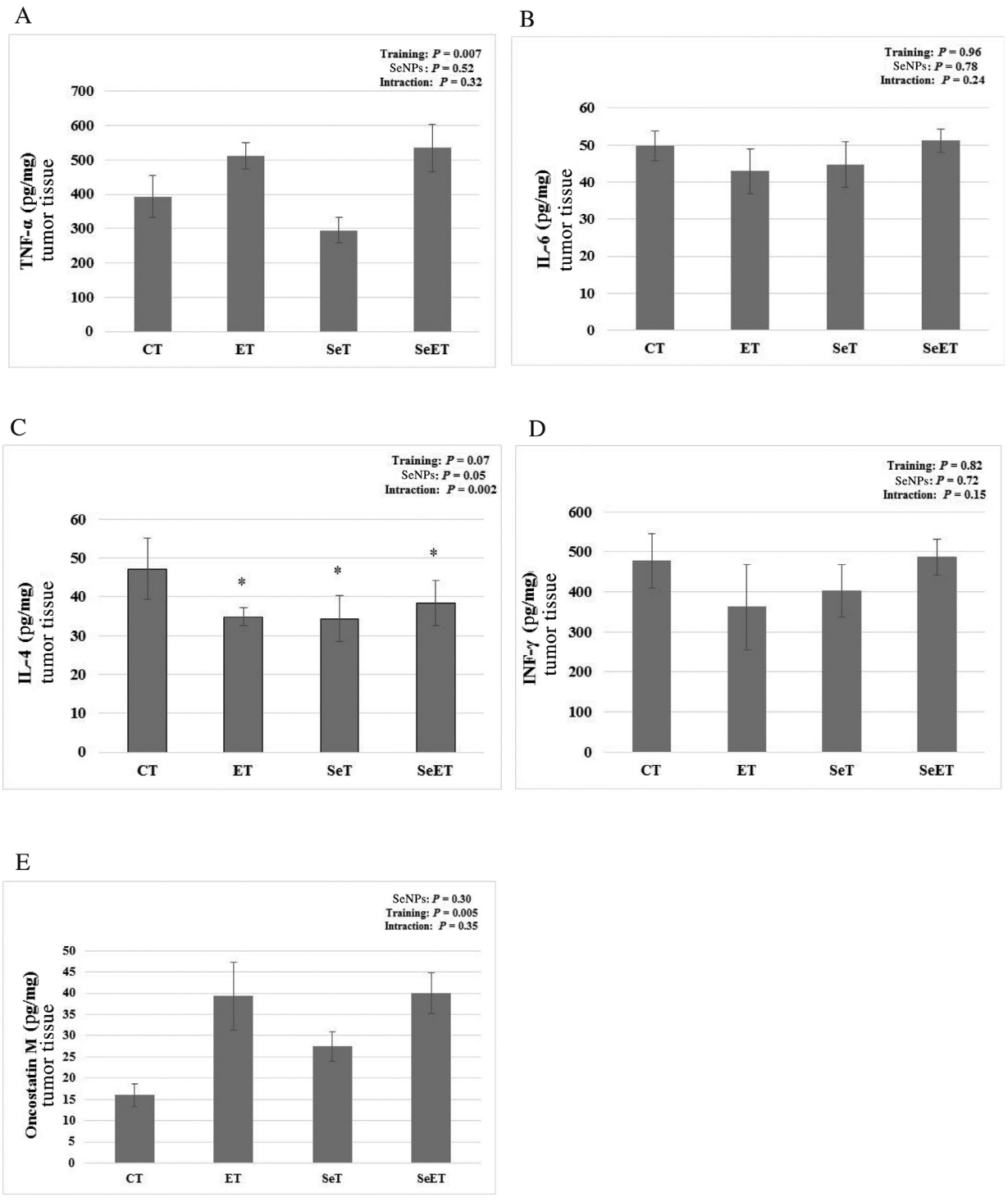

Fig. 2. Cytokine protein expression in tumor tissue. (**A**) TNF-α protein expression in tumor tissue (pg/mg tissue protein). (**B**) IL-6 protein expression in tumor tissue (pg/mg tissue protein). (**C**) IL-4 protein expression in tumor tissue (pg/mg tissue protein). (**D**) INF-γ protein expression in tumor tissue (pg/mg tissue protein). (**E**) OSM protein expression in tumor tissue (pg/mg tissue protein). Groups for mice are CT, control sedentary tumor-bearing; ET, trained tumor-bearing; SeT, tumor-bearing selenium nanoparticles; SeET, tumor-bearing selenium nanoparticles plus training. Results are expressed as mean ± SE. Results from the two-way analysis of variance are presented. N = 4–6 animals per group. For each panel, bars with superscripts are significantly different compared with CT group ($P < 0.05$). INF, interferon; OSM, oncostatin-M; SE, standard error; TNF, tumor necrosis factor.

Discussion

Physical exercise and antioxidant treatment have been shown to affect breast cancer survival [42]. The present study was undertaken to determine whether SeNP administration and AET affect tumor tissue cytokines. Also, the effects of SeNP supplementation and exercise training on Th1 and Th2 cytokine production in cultured spleen cells were evaluated. The main findings demonstrated that the

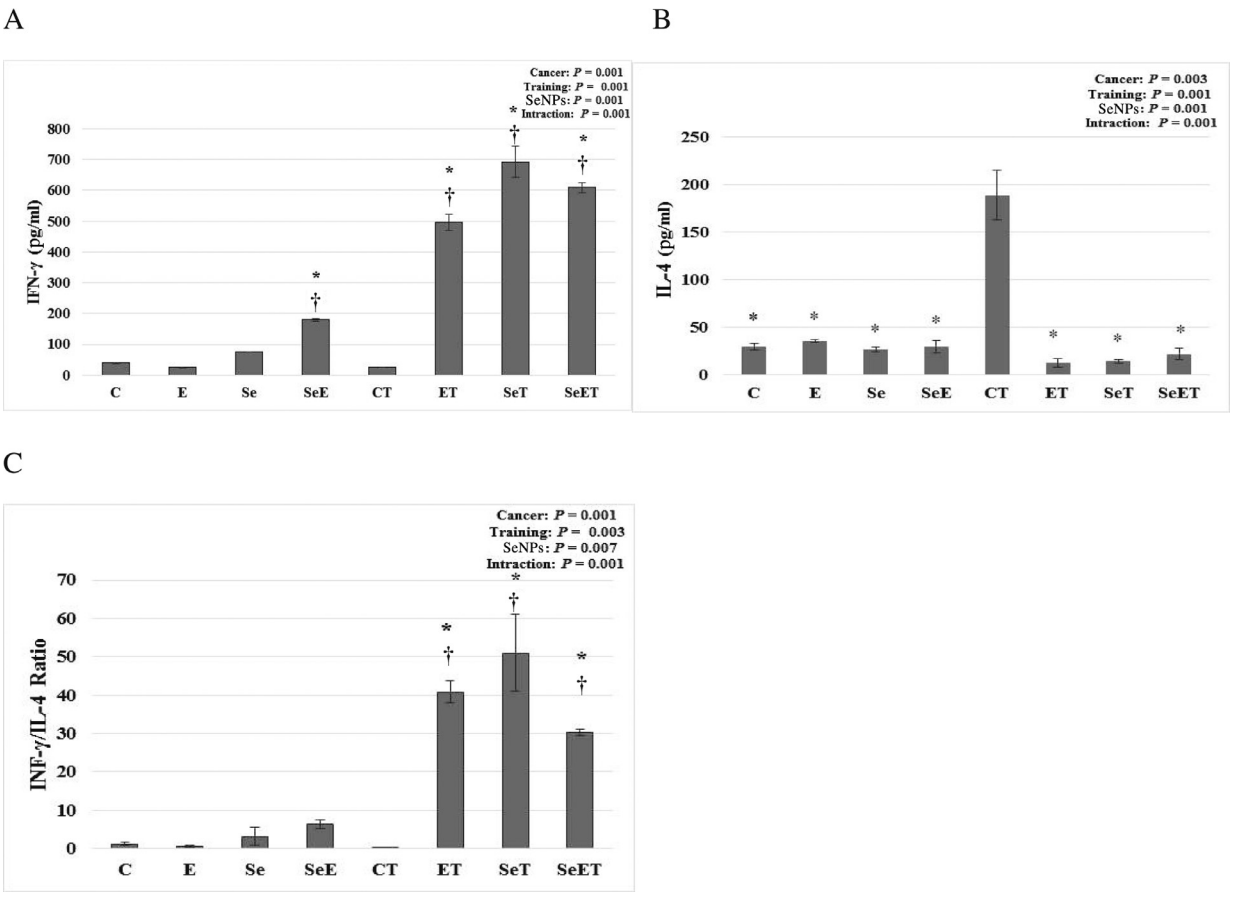

Fig. 3. Cytokine expression magnitude in splenocytes. INF-γ concentration in splenocytes (pg/mg). Groups for mice are C, control sedentary normal; CT, control sedentary tumor-bearing; E, normal trained; ET, trained tumor-bearing; Se, normal selenium nanoparticles; SeT, tumor-bearing selenium nanoparticles; SeE, normal nanoparticles plus training; SeET, tumor-bearing selenium nanoparticles plus training. Results are expressed as mean ± SE. Results from the three-way analysis of variance are presented. *Significantly different with control sedentary tumor-bearing group. †Significantly different with control sedentary normal group ($P < 0.05$). N = 2–4 animals per group. INF, interferon; SE, standard error.

combination of aerobic interval training with SeNP supplementation decreased tumor volume and promoted the Th1 immune phenotype, as measured by IFN-γ, in splenocytes of tumor-bearing mice. AET increased levels of OSM and TNF-α in tumor tissue, whereas SeNP supplementation decreased IL-4 levels tumor tissue.

Cytokine balance may indirectly reflect immune cell phenotype in the tumor microenvironment [9]. It has been suggested that a complex interplay between cancer cells and host immune responses has an important role in the progression of breast cancer [43,44]. The results of the present study indicate that aerobic interval training increased cytokines, which support host antitumor immune responses in breast cancer.

It is proposed that cytokines can activate antitumor immune responses or elicit immunotolerance in tumor microenvironment [44–46] and that high local levels of TNF-α cytokine in the tumor microenvironment can induce antitumor effects [47]. Also, monocytes and T cells are recruited into tumor microenvironments in inflammatory situations that occur particularly in the later stages of breast cancer and participate in cytokine induction in tumor tissue [9]. It was showed that T cells and monocytes stimulate OSM synthesis as an inflammatory cytokine [48].

In addition, AET and SeNP administration increased the Th1 immune phenotype, as measured by IFN-γ production by stimulated splenocytes in the present study. Th1 cells have been shown to play a major role in antitumor immune responses and are modulated by proinflammatory cytokines [49]. It is proposed that Th1 immune cells polarize macrophage to produce proinflammatory cytokines in tumor tissue [50]. However, the tumor microenvironment often inhibits activated T cells from entering tumor tissues [51]. TNF-α and OSM levels in tumor tissue and Th1 cytokine production in splenocytes increased after exercise training in the present study. It is possible that positive effects of exercise training on immune responses induce more effective T cells into the tumor microenvironment [41].

Moreover, exercise training and SeNP administration decreased IL-4 levels in tumor in breast cancer in the present study. Th2 lymphocytes are believed to be a major source of IL-4 in the tumor microenvironment, leading to activation of macrophages and resulting in more aggressive tumor behavior [10,11]. Changes in tumor cellular metabolism, especially shifting tumor metabolism from glycolytic to oxidative phosphorylation, is an efficient mechanism to decrease tumor growth [52]. It is proposed that energy demand increases during exercise cellular metabolism in tumor tissue may lead to an antitumor effects in the tumor microenvironment [53,54]. Also, alterations in tumor metabolites, such as increasing lactate production, have been hypothesized to inhibit antitumor immune responses [55–57]. It has been suggested that increasing IL-4 levels increased glucose metabolism in 4 T1 cancer cells [43]. Affecting tumor metabolism by aerobic interval training could be one mechanism for changing IL-4 levels in the tumor microenvironment, as observed in the present study, and could lead to an improvement in antitumor immune responses.

Taking into consideration that a tumor-directed immune response involves cytolytic CD8-positive T cells, Th1 and natural killer (NK) cells appear to protect against tumor development and progression [51]. Some studies propose that the immunomodulatory effects of SeNPs are more obvious in specific parts of the immune response such as Th1 cytokine production in cancer [30]. In addition, AET has greater effects on other parts of the immune response, such as NK cells [58]. More studies in this field are necessary to determine the relationship between tumor tissue and immune responses after AET.

Animal models are effective for examining the effects of different types, intensity, and duration of exercise on tumorigenesis in different stages. Until now, the most exercise training recommendations for patients with cancer have been moderate intensity [59]. Some positive effects from this kind of exercise training, such as antiinflammatory effects, have been observed [16,41]. However, it is proposed that shifting to an antiinflammatory state may be favorable for cancer prevention; on the other hand, it may also result in a hampered response of immune cells in the tumor microenvironment [60]. Short bouts of aerobic training at a vigorous intensity interspersed with low-intensity recovery and its effectiveness on systemic and local antitumor immune responses can be considered in the future investigations on breast cancer and exercise training.

It has been proposed that the protective effects of exercise training on tumor development and progression occur through exercise-induced changes, such as modulation of immune function [61]. Nevertheless, these positive effects must be clarified because the immune responses in the literature are controversial.

Conclusion

The present study showed that AET and antioxidant supplementation can be a promising strategy for decreasing tumor volume and increasing antitumor immune responses. The increased Th1 cytokine production in splenocytes after AET and supplementation with SeNPs as well as the positive effects of aerobic interval training in inducing antitumor cytokines in tumor tissue support this statement.

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