Original Article

The Effects of 4-Week Aerobic Exercise on the Levels of CCl2, CCl5, and their Respective Receptors in Female BALB/C Mice Suffering from Breast Cancer

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Abstract

Background: The studies reported that chemokines Chemokine (C-C motif) ligand 2 (CCL2) and Chemokine (C-C motif) ligand 5 (CCL5) have tumor-promoting roles in breast cancer (BC). The aim of the present study was to evaluate the effect of 4 weeks of continuous aerobic exercise (AE) on chemokines CCL2 and CCL5 and their relative receptors in animal model of human BC.

Materials and Methods: BALB/c mice were divided randomly into four groups included cancer control (CC) and three other groups. The total duration of the experiment was 14 weeks, including 2 weeks of familiarization of mice with treadmills and three of 4-week periods of experiment. Tumor inoculation and formation were performed in the second 4-week period. Group 1 received AE in the first 4-week, Group 2 received AE in the second 4-week and Group 3 in the third 4-week.

Results: The CCL2 was reduced significantly in Groups 1, 2, and 3 compared to control ($F_{3,12} = 4705$, $P = 0.0001$). In terms of CCL5, a significant decrease was seen only between Group 3 and control. Western blot results showed a significant reduction in C-C chemokine receptor Type 2 (CCR2) between Group 1 versus CC and Group 2 versus CC ($F_{3,20} = 1.812$, $P = 0.004$). In terms of C-C chemokine receptor Type 5 (CCR5) a significant decrease was observed between Group 2 versus control and Group 3 versus control ($F_{3,20} = 273.3$, $P = 0.042$), ($P = 0.004$).

Conclusion: It can be concluded that 4-week AE significantly reduces the chemokines CCL2 and CCL5 and their respective receptors levels CCR5 and CCR2 in different stages, and it may have an inhibitory effect on tumor growth.

Keywords: Breast cancer, breast neoplasm, breast tumor, chemokines, exercises, physical activity

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Introduction

According to the WHO statistics, breast cancer (BC) is the first leading cause of cancer-related deaths among women worldwide, with around 2 million women affected annually. In 2018, it is estimated that approximately 15% of all cancer deaths among women were due to BC.[1] Many clinical trials and meta-analyses investigated the effect of physical activity (PA) on BC. They found that pre and postdiagnosis PA was inversely associated with breast-cancer mortality and higher survival rate among BC survivors. All-cause mortality can be decreased by 67% in BC women with PA.[2-5] Aerobic exercise (AE) is also used to improve chemotherapy and/or radiation therapy-related fatigue in patients after cancer treatment (T);[6] however, still

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the molecular and biological mechanisms underlying this association are not studied enough.[7] There is an emerging field nowadays known as exercise–oncology which aims to investigate the biological mechanisms by which AE affects cancer development, progression, and/or metastasis.[8]

Many studies reported that chemokines Chemokine (C-C motif) ligand 2 (CCL2) (monocyte chemoattractant protein 1) and Chemokine (C-C motif) ligand 5 (CCL5) (regulated on activation, normal T-cell expressed and secreted) play important role in BC development.[9] These chemokines and their related receptors, C-C chemokine receptor Type 2 (CCR2) and C-C chemokine receptor Type 5 (CCR5), also found to serve as the mediators of chronic inflammation and cancer initiation/progression.[10] Studies have shown that inflammation is one of the main contributors to tumor development.[11] The progressive inflammatory situation also may cause poor prognosis of cancer in patients.[12] A study showed that extracellular *in vivo* levels of CCL2 and CCL5 were 3-5 times higher in the cancerous tissue of women with BC than normal breast tissue.[13] So far, no study investigated the effect of AE on CCL2 and CCL5 and their related receptors in BC. Therefore, in the present study, we aimed to study the changes in the expression levels of CCL2, CCL5, CCR2, and CCR5 in animal models with human BC after 4-week AE during different stages of cancer development including before tumor formation, simultaneously with tumor formation, and after tumor formation.

Materials and Methods

Animal

The present study is an experimental research with animal model which was performed in accordance with the guidelines and study protocols of the Animal Ethics Committee of the Isfahan University of Medical Sciences (ethics number: IR.UI.REC.1399.004). Female BALB/C wild-type mice (4–5 weeks old, n = 24, average weight of 18 ± 2 g), were kept six per cage in standard laboratory conditions (23°C ± 1°C; 50% ±3 humidity; 12:12 light-dark cycle). Animals were randomly divided into four groups, i.e., one cancer control group (CC) and three experiments Group 1, 2, and 3.

Cell culture and tumor formation

4T1 cells lines were purchased from the Iranian Genetic Resources (Tehran, Iran). The cells were maintained in RPMI 1640 medium (GIBCO, USA, 21875091) supplemented with heat inactivated 10% fetal bovine serum (FBS) (GIBCO, USA, 10099141), 120 mg/l penicillin, and 200 mg/l streptomycin. Cells were incubated at 37°C, 7.5% CO_2, and full humidity and were subcultured at 75% confluency (every 5 days) to maintain the cell in constant exponential growth.

Then, mice were anesthetized using the appropriate dose of ketamine and xylazine (10 mg to 1 mg). Two million cells were injected subcutaneously into the upper right thigh of the female BALB/C wild mice. About 10–20 days after the injection of cancer cells (2nd 4-week period of AE) the tumor was palpable in the injected area [Figure 1].[14]

Figure 1: Tumor inoculation and separation of tumor from surrounding tissue

Aerobic exercise procedure

Afterward, mice were introduced to doing the activities on treadmills for 2 weeks (6–18 m/min for 20 min). The experimental groups received 4 weeks of AE in different periods whereas the control group did not receive any exercises during these 4 weeks.

In all groups including CC, Group 1, 2, and 3 the tumor was formed in 2nd 4-week period. Group 1 received AE at the first 4 weeks before tumor formation, Group 2 received AE at the second 4-week at the same time with tumor formation, and Group 3 received AE at the third 4-week period. The AE was performed with intensity of medium oxygen consumption as 40%–50% at 18 m/min speed (fixed speed) for 40 min 5 times per week.

Sampling and analysis (enzyme-linked immunosorbent assay and Western blot)

After the last session, animals were anesthetized using ketamine + tyrosine injection, and their blood samples were collected from the large underlying vein. Plasma (1–2 ml) was separated after centrifugation at 10,000 g, 5460 rpm (for 10 min), and stored at −80°C until analysis. Plasma CCL2 and CCL5 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Minneapolis MN R and D Systems).

Tumors were separated from the surrounding tissues and dermis and were homogenized with protein extraction solution (PRO-PREP™, Intron Biotechnology, Seoul, Korea) and 10 mg/mL proteinase inhibitor (Roche) before being incubated at 4°C with mild agitation for 2–3 h. Samples were centrifuged (Eppendorf 5415 R centrifuge) at 4°C at 12,000 rpm for 10 min. The supernatant containing the protein was extracted and used for Western blot. Protein concentration was determined using the Bradford protein assay. Twenty micrograms of protein per sample were separated on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Blots were blocked for 1 h at room temperature using blocking solution (Roche) and then incubated with primary antibody, CCR2 (anti-mouse or human) and CCR5 (anti-mouse or human) (all 1:1000; Abcam), overnight at 4°C along with a control (β-actin 1:1000; Abcam) followed by washing with 0.1% TBST solution (5 min vigorous shaking

on three occasions). The paper was next incubated with HRP-conjugated secondary antibody for 1 h (1:3000; Abcam) followed by coating with the ECL kit and observed using radiology film. The ImageJ software was used to convert the Western blot images into quantitative data.

Statistical methods

All statistical analyses were performed using the SPSS, version 22 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to evaluate the intergroup variations after ensuring the data normality and homogeneity of variances using the LT. In addition, the least significant difference *post hoc* test was applied to examine the differences between groups. When the LT was significant, there was no homogeneity of variances using the F-adjusted Welch test; then, the GaussHavel *post hoc* test was conducted. Significance level was considered $P \leq 0.05$.

Results

Table 1 shows the mean and the standard deviation of tumor volume CCL2, CCL5, CCR2, and CCR5 levels in control (CC), Group 1, Group 2, and Group 3.

ANOVA analysis of ELISA results showed that there is a significant difference between Group 1, 2, and 3 versus CC in terms of CCL2 levels; therefore, CCL2 has been decreased significantly in all groups compared to control ($F_{3,12} = 4{,}705$, $P = 0.0001$), but the difference between the other Groups (1 and 2 vs. 3) was not significant. In terms of CCL5 expression, the ELISA results showed a significant difference between Group 3 versus CC and Group 1 versus 3; therefore, CCL5 has been decreased significantly in Groups 1 and 3 compared to control. The difference between groups in terms of CCL5 level was not significant [Figure 2].

The results of Western blotting for CCR2 showed a significant difference between Group 1 versus CC and Group 2 versus CC ($F_{3,20} = 1.812$, $P = 0.004$). In term of CCR5 level, a significant difference was observed between Group 3 versus CC, and Group 2 versus CC ($F_{3,20} = 273.3$, $P = 0.042$), ($P = 0.004$) [Figures 3 and 4]. There was no significant difference between the other groups [Table 1].

Discussion

In this study, we have identified the association between inflammatory chemokines CCL2 and CCL5 and their respective receptors and PA in BC along different stages of tumor formation. Our results showed that 4-week adjuvant endocrine therapy (AET) interventions cause a significant decrease in CCL2 levels in groups compared to the control. The relative receptor of CCL2, i.e., CCR2 showed a significant reduction followed by 4-week AE before tumor formation and simultaneously with tumor development. In terms of CCL5 and its related receptor (CCR5) a significant decrease was seen only in Group 3 for CCL5 and between Groups 2 and Group 3 for CCR5. It seems that AE, even with moderate intensity and for a short period (4 weeks), causes a significant decrease in CCL2 and CCL5 chemokines and their related receptors. Because these two chemokines (CCL2 and CCL5) have a tumor-enhancing impact, it can be deduced that AE can limit tumor formation and even growth. Based on our results which showed decreased CCL5 and its respective receptor CCR5 levels followed by AET in the last 4-week period after tumor formation and parallel to tumor progression, it might be possible that 4-week AET act as an inhibitory and therapeutic agent in tumor growth and development stage.

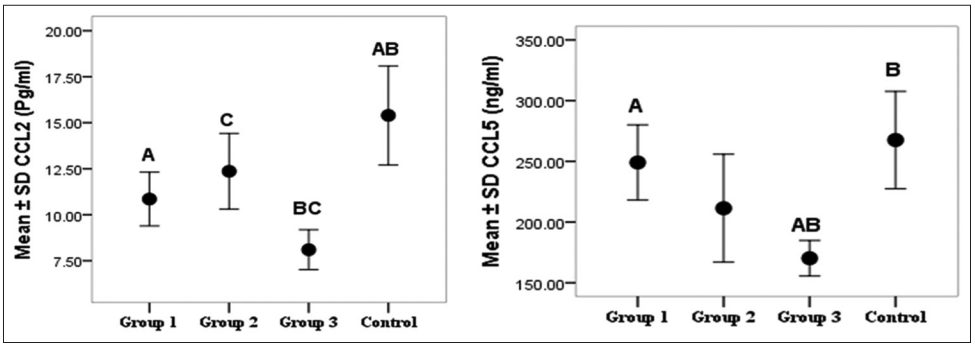

Figure 2: Chemokine (C-C motif) ligand 2 and Chemokine (C-C motif) ligand 5 levels in the study Groups; AA, BB, CC, and $P < 0.05$

Table 1: Mean and standard deviation values of the research variables in four groups

	1	2	3	CC	*F*	*P*
CCL2 (pg/ml)	10.85±1.79[a]	12.36±2.52[e]	8.10±1.33[c]	15.40±3.29	10.025	0.001
CCR2 fold	0.6835±0.1868[a]	0.6270±0.1161[b]	0.8488±0.2558	1.00±0.00	6.007	0.004
CCL5 (ng/ml)	249.09±37.93[d]	211.38±54.43	170.20±17.93[c]	267.58±49.07	6.262	0.004
CCR5 fold	0.9139±0.1094	0.7339±0.2482[b]	0.8108±0.0479	1.00±0.00	4.297	0.017

[a]$P<0.05$ 1 versus control; [b]$P<0.05$ 2 versus control; [c]$P<0.05$ 3 versus control; [d]$P<0.05$ 1 versus 3; [e]$P<0.05$ 2 versus 3. CCL2, CCR2, CCL5, CCR5, Group 1, Group 2, Group 3, CC. Data are presented as mean±SD. CC: Cancer control, SD: Standard deviation, CCL2: Chemokine (C-C motif) ligand 2, CCR2: C-C chemokine receptor type 2, CCL5: Chemokine (C-C motif) ligand 5, CCR5: C-C chemokine receptor type 5

In fact, inflammation is a key factor in the growth and spread of BC and other types of tumors. Chemokines can target tumor stem-like cells and stromal cells both directly and indirectly. CCL2 and CCL5 can promote tumor metastasis.[15] They can cause monocytes to secrete matrix metalloproteinase 9, which permits tumor cells (TC) to extravasate by destroying the matrix. CCL2 and CCL5 can also help cancer cells proliferate, survive, and move around.[16] Therefore, inhibition of inflammation through the reduction in inflammatory chemokines such as CCL2 and CCL5 and their respective receptors CCR2 and CCR5 levels could be a possible goal in preventing tumor progression and cancer T. In a study by Chiu *et al*., it was shown that the CCL2/CCR2 signaling pathway facilitated migratory and invasive activities in high-grade human bladder cancer cells and that inhibiting the route reduced migration and invasion. Their data show that the CCL2/CCR2 pathway could be a promising candidate for reducing bladder cancer metastasis.[17] Increases in PA have been linked to lower circulating concentrations of pro-inflammatory cytokines and greater circulating concentrations of anti-inflammatory cytokines as a result of AE.

Soria *et al*. have established a positive correlation in BC between even benign BC or aggressive BC and CCL2 levels and also between the aggressiveness of the tumor and CCL5 levels. Their data imply that CCL2 and CCL5 expression coordination is critical for cancer progression.[18] Although activation of CCL2/CCR2 signals does not significantly affect cell growth, it causes cell migration and invasion through activation of kinase C protein and phosphorylation and tyrosine in paxillin. Blocking CCL2 and CCR2 with shCCL2 inhibits cell migration through CCL2/CCR2.[19]

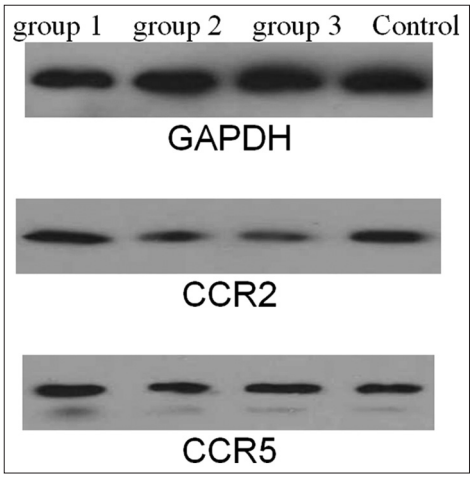

Figure 3: Western blot images

In this study, an increase in chemokines and its receptors was observed in the CC group, which is in line with other studies. Unlike normal breast epithelial duct cells, breast TC expresses high levels of CCL2 and CCL5. These two chemokines alter the balance between the subtypes of leukocytes at the tumor site, as well as increase the use of monocytes, the presence of destructive tumor-associated macrophages (TAM) in the tumor, and inhibit the potential T-cell antitumor activity. In addition, CCL2 mainly enhances angiogenesis, and both chemokines act directly on TC to enhance their ability to be invasive. TCs are promoted by the migratory phenotype, thus supporting invasion and metastasis. These activities of CCL2 and CCL5 exacerbate the presence of TAM-induced tumor-stimulating factors at the tumor site (growth factors, ECM-destroying enzymes, immune system suppressants, and angiogenesis mediators) and suppress the immune system. These chemokine functions cause tumor growth at the site of the primary tumor as well as metastatic spread.[20,21]

One of the limitations of this study was the lack of positive control in healthy individuals. Therefore, the effect size of mentioned parameters in CC and experimental groups compared to normal BC cannot be obtained. Small sample size, short time duration of exercise, and the fixed speed of treadmill during the study are numbers of factors that limit our study.

Conclusion

The duration of 4 weeks of AE decreases CCL2 and CCl5 levels and their respective receptors CCL5 and CCR5 levels in animal models with human BC. More studies are needed with regard to reveal the details of the molecular mechanism of exercise therapy's effect on cancer.

Ethical approval

The experimental procedures had already been confirmed by the Isfahan University Ethics committee.

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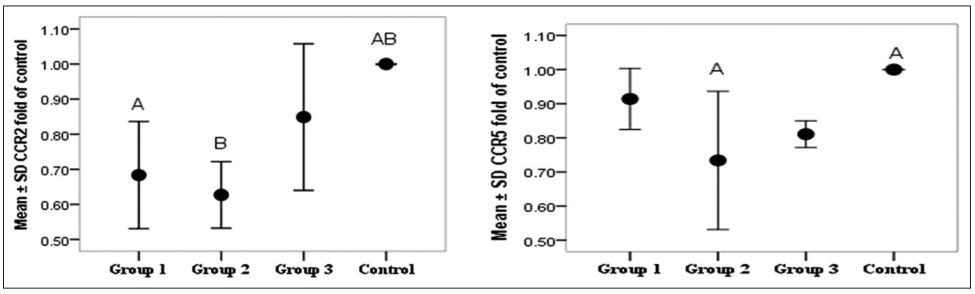

Figure 4: C-C chemokine receptor Type 2 and C-C chemokine receptor Type 5 levels in the study Groups; AA, BB, and $P < 0.05$

physicians, and other personnel at Isfahan University of Medical Sciences.

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Conflicts of interest

There are no conflicts of interest.

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