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Original Article

Effects of 8-Week Noncontinuous 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: Recently, the importance of chemokines and their receptors in carcinogenesis and the protective role of aerobic exercise in primary cancer development and progression is highlighted. Based on the facts that endurance exercise may result in hypoxia condition, and in addition, the effect of exercise therapy on the levels of CCL2, CCR2, and their related receptors in breast cancer (BC) model has not been investigated so far, therefore we aimed to evaluate the effect of eight-week noncontinuous aerobic training on the levels of CCL2, CCL5, and their related receptors; CCR2 and CCR5 in female BALB/C mice with BC. **Methods:** Thirty-two BALB/C mice (4–5 weeks old) were randomly divided to four 8-member groups of control and experimental. The experimental group received 8 weeks of noncontinuous aerobic exercises (AEs) while the control group did not receive any exercises during these 8 weeks. After last of experiment, 5 ml of blood was taken from each rat's lower inferior vein. The plasma levels of CCL2 and CCL5 were measured by ELISA and CCR2 and CCR5 by western blot. Tumor volume also measured in each step. Data were analyzed using the ANOVA test and the SPSS v. 0.24 Software. **Results:** After 8 weeks of participation in noncontinuous AEs, a statistically significant decrease was made between the control and experimental groups in terms of CCL2, CCL5, and CCR2 levels, as well as tumor volume. However, there was no significant difference between groups in terms of CCR5 level. **Conclusions:** It can be concluded that the 8 weeks of noncontinuous AEs did not result in CCR5 reduction while resulting in a statistically significant decrease in CCL2, CCL5, CCR2, and tumor volume.

Keywords: *Breast cancer, chemokine, CCL2, CCR2, CCL5, CCR5, exercise*

Introduction

According to many clinical trials and meta-analysis, the aerobic exercise (AE) has the potential to improve the health of cancer survivors and prevent the tumor in women with breast cancer (BC). It has been reported that all-cause mortality can be decreased by 67% in BC women followed by physical activity (PA).[1-4] However, evidence is still lacking on the effectiveness of different type, and time of exercise programs. For example, it has been reported that having 300-min walk per day as moderate-intensity PA will reduce the risk of BC mortality.[3] Although there are studies on evaluating the association between carcinogenesis and PA either resistance or continuous AE, still there is a paucity of experiments and studies to find out whether noncontinuous AE can also have a positive and protective effect on cancer. It is possible that continuous exercising may result in hypoxia condition.[5] Low level of oxygen which is called hypoxia is a common feature of all tumors that can change the cell behavior and is associated with extracellular matrix remodeling and increased chance of migratory and metastatic and resistance to treatment.[6]

The exact molecular mechanisms underlying the protective effect exercise therapy in patients with cancer are still not well understood. According to the studies, inflammation is one of the important contributors to cancer development and progression[7] and the progressive inflammatory situation causes poor prognosis of cancer-affected patients.[8] Studied in healthy individuals showed PA as regular exercise exerts anti-inflammatory elements.[9,10] Few studies have evaluated the effects of exercise on inflammatory markers after a cancer diagnosis.

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How to cite this article: Esmailiyan M, Kargarfard M, Esfarjani F, Vaseghi G. Effects of 8-week noncontinuous aerobic exercise on the levels of CCL2, CCL5, and their respective receptors in female BALB/C mice suffering from breast cancer. Int J Prev Med 2022;13:55.

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Access this article online

Website: www.ijpvmjournal.net/www.ijpvm.ir

DOI: 10.4103/ijpvm.IJPVM_93_20

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A number of chemokines such as CCL2 and CCL5 and their receptors, CCR2 and CCR5, reported as mediators of chronic inflammation and consequently tumor development and progression.[11,12] A study showed that CCL2 and CCL5 levels are 3–5 times higher in the cancerous tissue compared to normal breast tissue of BC-affected women.[13] Previously effect of 8 weeks of noncontinuous AE was studied in rats with cardiovascular diseases,[5] so we aimed to study the effect of 8 weeks of noncontinuous AE on the levels of CCL2 and CCL5 and their respected receptors, that is, CCR2 and CCR5, in animal model suffering from BC, and to the best of our knowledge our study is first.

Methods

The present study is an experimental research with an animal model. This study investigates the changes in some parameters and factors resulted from applying a noncontinuous AE program in three different experimental groups suffering from BC (n = 8/group), a control group suffering from BC (n = 8). All four groups were consisted of female BALB/C mice (4–5 weeks old, 18 ± 2 g), purchased from Pasteur Institute (Tehran, Iran). Animals were kept in standard laboratory conditions (23 ± 1°C; 50% ± 3 humidity; 12:12 light–dark cycle). In this study, animals were provided with free access to water and food. All experiments were 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). It is worth mentioning that the present research has also made use of rodent treadmills for exercise protocols.

Cell culture

4T1 cells lines were purchased from Iranian Genetic Resources (Tehran, Iran). The cells were maintained in RPMI 1640 medium (GIBCO, USA, 21875091) supplemented with heat inactivated 10% fatal bovine serum (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) in order to maintain the cell in constant exponential growth.

Noncontinuous AE training

Afterward, the subjects of the experimental groups were introduced with doing the administered activities on treadmills for 2 weeks (6–18 m/min for 20 min). The experimental group received 8 weeks of noncontinuous AEs alternatively in different 4-week periods while the control group did not receive any exercises during these 8 weeks. Our three experimental groups were (1) before–during cancer formation, (2) during–after cancer formation, and (3) before–after cancer formation.

The process of cancer formation was performed in the second 4-week period of the experiment. At the first four weeks, groups 1 and 3 received AE while groups 2 and control remained inactive. At the second four-week course, groups 1 and 2 received exercise simultaneously with cancer cells injection and tumor formation. At the third four-week period, groups 2 and 3 received AE and groups 1 and control remained immobile. Table 1 shows the overall schedule for noncontinuous AE training protocol in the present study. 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.

After the last session, animals were anesthetized using ketamine + zyrosin injection, and their blood samples were collected from the large underlying vein. Plasma was separated after centrifugation (10 min at 10,000g) and then stored at −80°C until analysis. Plasma CCL2 and CCL5 levels were measured using the ELISA. The tumors were separated from the surrounding muscles and dermis and subjected for western blot analysis of CCR2 and CCR5.

Table 1: The 8-week program of noncontinuous aerobic exercise

Training period	Period (weeks)	Speed (m/min)	Time (min)	Repeat (days per week)	Activity
Introduction	2	6-18	20	5	Introduction to treadmill
Before- During cancer	8	18	40	5	The first 4-week period: aerobic exercise, the second 4-week period: Tumor formation and aerobic exercise, the third 4-week period: detraining
During-After cancer	8	18	40	5	The first 4-week period: detraining, the second 4-week period: Tumor formation and aerobic exercise, the third 4-week period: aerobic exercise
Before-After cancer	8	18	40	5	The first 4-week period: aerobic exercise, the second 4-week period: Tumor formation and detraining, the third 4-week period: aerobic exercise

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Statistical methods

All statistical analyses were performed using SPSS, version 22. One-way ANOVA was used to evaluate the intergroup variations after ensuring the data normality and homogeneity of variances by using the LT. In addition, the LSD post-hoc test was applied in order to examine the differences between groups. When the LT was significant, there was no homogeneity of variances by using the *F*-adjusted Welch test; then the Gauss–Havel post-hoc test was conducted. Significance level was considered $P \leq 0.5$.

Results

Table 2 shows the mean and standard deviation of TV, CCL2, CCL5, CCR2, and CCR5 and the weight difference in our study.

One-way ANOVA results showed that there was a significant difference in CCL2 levels between groups ($F_{3,20}$ = 30.124, P = 0.001). After conducting the Tukey post-hoc test (TPHT), there was a significant difference between group 1 (before–during cancer formation) and control (P = 0.001); group 2 (during–after cancer formation) and control (P = 0.001); and group 3 (before–after cancer formation) and control (P = 0.001). However, the difference between the other groups was not significant.

One-way ANOVA results showed that there was a significant difference in CCL5 levels between groups ($F_{3,20}$ = 15.948, P = 0.001). After conducting the TPHT, a significant difference was observed between group 1 (before–during cancer formation) and control (P = 0.001); group 2 (during–after cancer formation) and control (P = 0.003); and group 3 (before–after cancer formation) and control (P = 0.001). However, the difference between the other groups was not significant [Figure 1].

ANOVA results showed that there was a significant difference in CCR2 expression fold between groups ($F_{3,20}$ = 60.749, P = 0.001). After conducting TPHT, there was a significant difference between group 1 (before–during cancer formation) and control (P = 0.001); group 2 (during–after cancer formation) and control (P = 0.003); and group 3 (before–after cancer formation) and control (P = 0.001). However, the difference between the other groups was not significant.

ANOVA results showed that there was no significant difference in CCR5 between groups ($F_{3,20}$ = 0.691, P = 0.568). ANOVA results showed that there was a significant difference in terms of the TV between groups ($F_{3,20}$ = 9.041, P = 0.001). Based on the TPHT, there was a significant difference between group 1 (before–during

Table 2: Mean and standard deviation of investigated variables in the present study

	1	2	3	4	F	P
TV (mm3)	867.480±215.456[a]	819.938±181.754[b]	1069.740±424.103[c]	1642.278±344.864	9.041	0.001
CCL2 (pg.ml)	7.79±2.11[a]	6.17±1.19[b]	4.74±1.12[c]	15.40±3.29	30.124	0.001
CCR2 fold of control	0.6165±0.0635[a]	0.5678±0.0703[b]	0.2773±0.1608[c]	1.00±0.00	60.749	0.001
CCL5 (ng.ml)	200.37±11.60[a]	171.94±18.44[b]	169.67±15.91[c]	267.58±49.07	15.948	0.001
CCR5 fold of control	0.8710±0.2922	0.9889±0.2889	0.8597±0.1601	1.00±0.00	0.691	0.568
Δ W (g)	4.31±0.6	4.1±0.4	3.83±0.5	5.28±0.5		

Data are presented as Mean ± SD; TV, Tumor volume; 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; Δ Weight, Weight difference at the beginning of the study and at the end of the work ; 1 before-during, 2 before-after, 3during-after, 4control; [a]($P < 0.05$) 1 vs 4; [b]($P < 0.05$) 2 vs 4; [c]($P < 0.05$) 3 vs 4 (there was no significant difference between the groups)

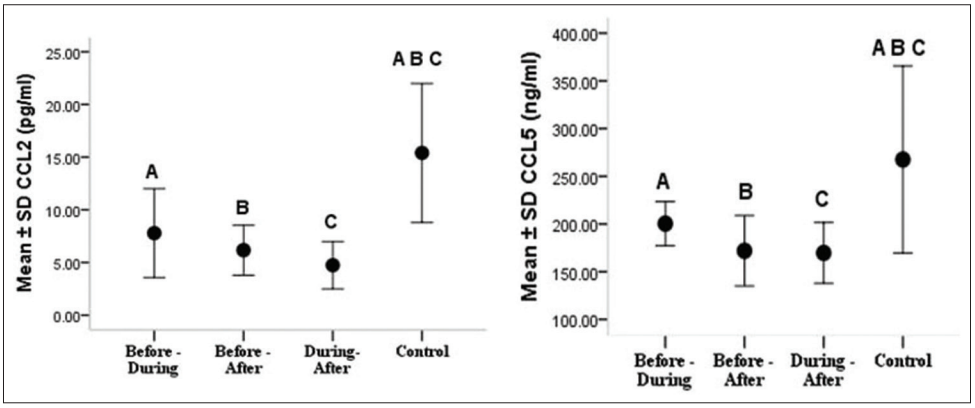

Figure 1: CCL2 and CCL5 levels in the study groups; AA, BB, CC, *P* < 0.05

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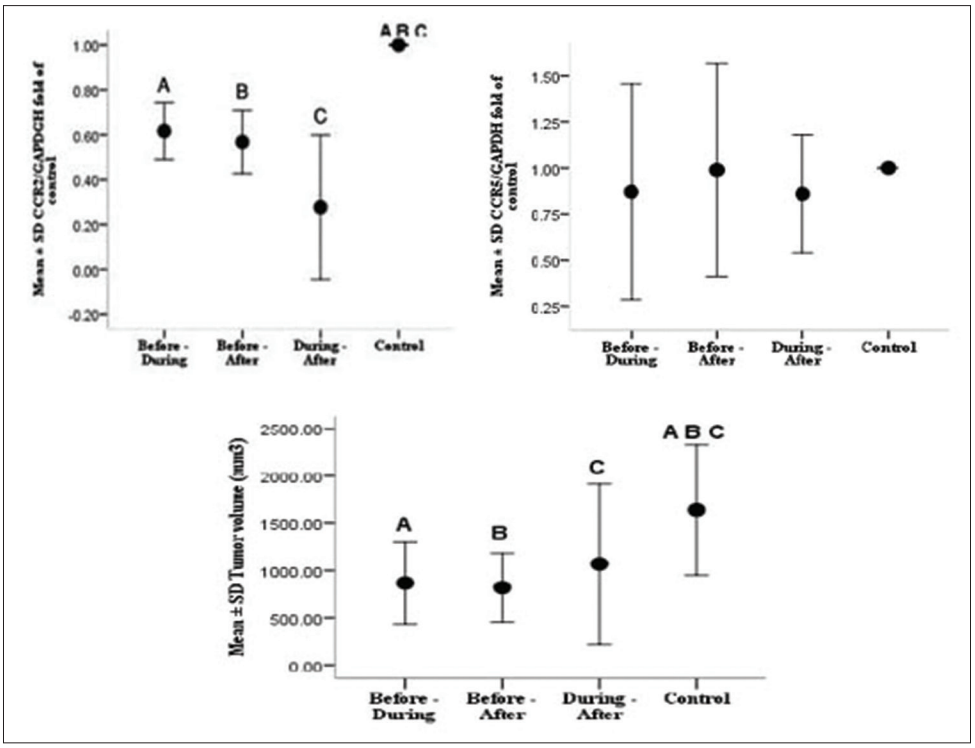

Figure 2: CCR2 and CCR5 levels and TV in the study groups; AA, BB, CC, $P < 0.05$

cancer formation) and control ($P = 0.020$); group 2 (during–after cancer formation) and control ($P = 0.002$); and group 3 (before–after cancer formation) and control ($P = 0.001$). However, the difference between the other groups was not significant [Figure 2].

Discussion

Results of the current study indicated that 8 weeks of noncontinuous AE result in a significant decrease in plasma levels of CCL2 and CCL5 and expression level of CCR2 and tumor volume compared to control group in the female mice with BC. The CCR5 level did not show any significant differences. Also, there were no statistically significant changes in term of CCL2, CCL5, and CCR2 levels intergroups.

Our results also showed that after exercise even as noncontinuous, the weight is reduced in all experimental groups. Weight gain, which mostly is due to fat accumulation and obesity, is associated with increased adipocyte size and macrophage infiltration, which, in turn, results in the altered levels of cytokines secreted from adipose tissue. Also decreased PA and increased abdominal adiposity are associated with persistent systemic low-grade inflammation which may result in cancer development or progression.[14] The present study is in line with the research done by Carlin *et al.*, which showed that PA could inhibit the expression of CCL2 and CXCL10 chemokine genes. So exercise can prevent the induction of chemokines by reducing inflammation and obesity and preventing the development of BC.[15]

In this study, CCL2, CCL5, and CCR2 were decreased in the all three groups: before–after cancer, before–during cancer, and during–after cancer compared to the control group, and the type of exercise program, including different exercise and rest times, did not make a difference. Although there was no significant difference between the groups (intergroups) in term of CCL2, CCL5, and CCR2 levels, however due to this significant reduction in cytokines and their receptors compared to the cancer control group, it can be concluded that exercise can have a positive protective effect against BC, even in the noncontinuous type as well as continuously. However, in order to compare the effectiveness and result of continuous aerobic and noncontinuous AE, it is necessary to conduct a separate study in this regard.

Some studies have shown that BC survivors have higher levels of circulating cytokines than healthy women.[16] Exercise may lead to reduction in circulating levels of proinflammatory cytokines and increase in circulating concentrations of anti-inflammatory cytokine. Exercise increases the sympathetic system. There is some evidence showing that in some patients with moderate-to-high levels of inflammatory markers, regular PA is associated with decreased proinflammatory cytokines in the bloodstream.[16-18] A similar study carried out by Qian *et al.* showed that CCL2–CCR2 signaling inhibited the inflammatory monocyte recruitment and metastasis, as well as enhancing the survival of tumor-bearing mice.[19] In review by Kang *et al.*, it was concluded that AE reduced the fasting insulin levels and inflammatory

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markers in the BC survivors and therefore these factors could be considered as prognostic markers for prevention of BC development through exercise.[20] Murphy *et al*. also showed that exercise training reduced plasma CCL2 and IL-6 concentration by decreasing the TV.[21] Chemokines enhanced the Jak-STAT or MAPK/ERK signaling pathway, as well as tumor cell proliferation, by activating tyrosine kinase receptors.[22] CCL5 knockdown induces cell proliferation by inducing the mTOR pathway, with mTOR pathway leading to the positive regulation of cyclin D1, c-Myc, and Dad-1 expression. An additional mechanism based on CCL5/CCR5 interaction can increase cell proliferation, glucose uptake, ATP production, and glycolysis, which is associated with the acidification of the extracellular environment.[23]

According to the results of the present study, no significant change was observed in CCR5 expression level compared to the control group. This could be attributed to the fact that CCR5 expression could promote tumor progression and metastasis process. One study showed that CCR5 expression specifically in stromal cells is associated with tumor metastasis.[24] The role of CCR5 in metastasis is reported for chondrosarcomas[25] and in oral cancer cells.[26] Therefore, given that tumor has formed for a short time in our study and has not yet reached the stage of metastasis and cell migration, it may be because the level of this receptor has not changed.

Conclusions

The results of this study showed that noncontinuous AE decreased the CCL2, CCL5, and CCR2 levels and the tumor volume significantly compared to control cancer group at different time intervals. There were no significant changes in CCR5 levels in this study compared to control. It can be concluded that noncontinuous AE has positive protective effect on cancer development and proliferation and can be considered as a kind of nonpharmacological intervention suitable for decreasing the rate of tumor growth.

Limitations

This study did not contain positive control of healthy subjects. Therefore, the effect size of mentioned parameters in cancer control 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 limit our study.

Acknowledgments

This study was financially supported by the University of Isfahan. The authors would like to thank the administrative staff, physicians, and other personnel at Isfahan University of Medical Sciences.

Financial support and sponsorship

The study was supported by Esfahan University.

Conflicts of interest

There are no conflicts of interest.

Received: 05 Apr 20 **Accepted:**14 Jul 20
Published: 05 Apr 22

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