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MicroRNA-206, let-7a and microRNA-21 pathways involved in the anti-angiogenesis effects of the interval exercise training and hormone therapy in breast cancer

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ABSTRACT

Aims: MicroRNAs (miRNAs) are the targeting signal-transduction pathways that can mediate tumorigenesis via their down and/or up-regulation. For example, miR-21 and miR-206 can effect on the tumor angiogenesis as an oncomir and a tumor suppressor, respectively.
Materials and methods: The present study is aimed to investigate the effects of the interval exercise training in combination with tamoxifen and/or letrozole on miR-21, miR-206 and let-7 as well as their underlying pathways in regard to tumor angiogenesis in sixty four mice with breast tumor. ELISA, immunohistochemistry, qRT-PCR assays were performed accomplish the study.
Key findings: The results showed that the tumor size was significantly declined in the exercise training, tamoxifen and letrozole groups compared to tumor group. Mir-206 and let-7 were up-regulated, and mir-21 expression was down-regulated in the exercise training compared to tumor group. Exercise training decreased the expression of ER-α, HIF-α, VEGF, CD31 and Ki67 in tumor tissue. The combination tamoxifen and/or letrozole with the exercise training could down-regulate the expression of ERα, miR-21, HIF-1α, TNF-α, CD31, Ki67 and VEGF, and up-regulate the expression of miR-206, PDCD-4, let-7 and IL-10 that led to reducing the angiogenesis and tumor growth.
Significance: Our results showed that miR-21, miR-206 and let-7a pathways may involve in the anti-angiogenesis effects of the interval exercise training with hormone therapy in mice model of breast tumor.

1. Introduction

According epidemiological studies the low levels of the physical activity increase the risk of different cancers such as breast [1] by several mechanisms including decreasing sex and metabolic hormones, inflammation and improving immune function [2]. In the other hand long-term exercise training attenuates mononuclear cell production of atherogenic cytokines (IL-1-alpha, TNF-alpha, and interferon gamma) while augments the production of atheroprotective cytokines (IL-4, IL-10 and TGF-b1) [3]. Regular exercise training can decrease the circulatory level of microRNAs such as miR-21 [4,5]. Holmes et al. showed that the exercise training had the more beneficial effects in women with the hormone-responsive tumors [6]. Moreover, increased expression of the human estrogen receptor alpha (ERα) is associated with an increased expression of miR-21 [7].

MicroRNAs are short noncoding RNA species of 22-nucleotide transcripts derived from primary mRNA transcripts of intergenic or intronic sources [8]. Among these miRNAs, mir-21 is well known as oncomir. Mir-21 increases tumor growth through up-regulation of the antiapoptotic protein Bcl-2, and anti-miR-21 inhibits tumor growth in vivo [9]. Moreover, mir-21 overexpression increases the hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression, and induces angiogenesis in human prostate cancer

Abbreviations: CD31, cluster of differentiation 31; CS, citrate synthase; E2, 17-β estradiol; EDTA, ethylenediaminetetra acetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER-α, estrogen receptor alpha; EX, exercise; HIF-1a, hypoxia-inducible factor 1-a; IHC, immunohistochemistry; IL-10, interleukin-10; LET, letrozole; MC4-L2, mouse mammary adenocarcinoma cell line; Mir, microRNAs; Mir-206, microRNA-206; Mir-21, microRNA-21; PDCD4, programmed cell death protein 4; TMX, tamoxifen; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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cells [10]. Moreover, some of miRNAs such as mir-206 have been shown act as tumor suppressors in breast cancer. Functional studies have shown that miR-206 targets ER-α and decreases its expression [11]. MiR-206 transfection appears to inhibit proliferation and invasion of lung cancer cells at least partly by regulating VEGF expression [12]. VEGF level can be an important independent tumor marker that will predict a poor efficacy of both tamoxifen and chemotherapy drugs used in advanced breast cancer [13]. VEGF participates directly in angiogenesis by the recruiting endothelial cells into the hypoxic and vascular tumor region [14] by stabilizing HIF-1α in the proteasome degradation pathway [15]. HIF-1α has been correlated to markers of the aggressive breast cancer cells such as Ki67 and ERα [16]. In this context, chronic and persistent inflammation contributes to cancer development and even predisposes to carcinogenesis [17], and inflammation is a critical component of tumor progression [18]. Tumor necrosis factor alpha (TNF-α) is an important pro-inflammatory cytokine in early events in tumors, regulating a cascade of cytokines, chemokines, adhesions, metalloproteases (MMPs) and pro-angiogenic activities [19]. Thus, TNF-α may be one of the ways in which inflammation acts as a tumor promoter. In contrast, IL-10 can inhibit tumor-induced angiogenesis and enhance the production of tumor-toxic molecules (e.g., nitric oxide), which can lead to tumor regression [20]. In addition, in vitro exposure of colonic intraepithelial lymphocytes to IL-10 resulted in down-regulation of miR-21, Let-7a, miR-101, miR-223, and miR-155 [21]. This may occur by inhibition of tumor suppressor and antiapoptotic pathways by via programmed cell death protein 4 (PDCD4) and tropomyosin1 (TPM1) [22,23]. Thus, the aim of the present study is to investigate the effects of 5 weeks interval exercise training in combination with the hormone therapy on miR-21, miR-206 and let-7a as well as their underlying pathways in regard to tumor angiogenesis in a typical animal model of breast cancer.

2. Materials and methods

2.1. Cell line and reagents

Mouse mammary adenocarcinoma cell line (MC4-L2) was obtained as a gift from Buenos Aires University, Argentina [24]. Ketamine and Xylazine (Sigma-Aldrich, USA), polyclonal mouse antiRat/Rabbit CD31 and Ki67 antibodies (DAKO Corporation, USA), mouse IL-10 ELISA kit (Abcam, Germany), mouse VEGF ELISA kit (Abcam, Germany), estradiol ELISA kit (Cayman Chemical, USA), and Trizol (life technology, USA) were purchased.

2.2. Cell culture

The mice mammary tumor cell line (MC4-L2) was grown in T75 flasks in DMEM/F-12 with 15 mM HEPES buffer, L-glutamine, penicillin 100 μg/ml, streptomycin 100 μg/ml, 10% FBS (Gibco BRL, Life Technologies) and 10 nM Medroxy Progesterone Acetate (Sigma Chemicals, Ontario, Canada). The cells were detached by 0.025% trypsin, rinsed with PBS and enzymatically neutralized by 10% FBS and finally centrifuged in 1200 rpm for 3–5 min. The cells viability was determined by Trypan blue and hemocytometer, respectively [5,24].

2.3. Animals

Animal studies have conducted according to the relevant national and international guidelines of Weatherall report, and Institutional Animal Care and Use Committee (IACUC) of Tehran University of Medical Sciences. Inbred female BALB/c mice (6–8 weeks old), purchased from Pasteur Institute of Iran, maintained under 12-hour dark and light cycle, with free access to food and water.

2.4. Tumorigenicity

MC4-L2 cells were trypsinized and re-suspended in 10-fold excess culture medium. After centrifugation, the cells were re-suspended in a serum-free medium. The prepared cells (1×10^6 cells in a final volume of 0.1 ml) were inoculated in the right inguinal flank near to back of animals under anesthesia with Ketamine (100 mg/kg) and Xylazine (10 mg/kg).

2.5. The study design

Sixty four mice were equally randomized to one of eight groups (N = 8): (I) control (C); the healthy animals with neither tumor nor exercise training protocol, (II) exercise training (EX); the animals under the interval exercise training protocol for 5 weeks without tumor cell injection, (III) tumor (T); the animals with breast tumor, (IV) tumor plus exercise training (T + EX); the animals with breast tumor underwent 5 weeks of the interval exercise training protocol after tumor establishment, (V) tumor plus tamoxifen (T + TMX); TMX (5 mg/kg daily) was given via oral gavage for 2 weeks after tumor establishment, (VI) tumor plus tamoxifen and the interval exercise training (T + TMX + EX); the animals with breast tumor underwent 5 weeks of the interval exercise training protocol plus tamoxifen after tumor establishment (VII) tumor plus letrozole (T + LET); LET (5 mg/kg, daily, oral) was given via oral gavage for 2 weeks after tumor establishment, and (VIII) tumor plus letrozole and exercise training (T + LET + EX); the animals with breast tumor underwent 5 weeks of the interval exercise training protocol plus letrozole. At the end of study, all animals were euthanized 48 h after the last session of the interval exercise training.

2.6. Interval exercise training protocol

Following tumor establishment, the animals were equally divided into the mentioned groups, and the body weight and tumor volume were measured. Prior to the initiation of the exercise training, the mice were assigned to the treadmill for 5 days. Acclimation entailed

Table 1
Nucleotide sequences of the primers used for real-time RT-PCR.

Gens	Forward sequence	Reverse sequence	NCBI
miR-21	TAGCTTATCAGACTGATGTTGA	–	NR_029738
miR-206	TGGAATGTAAGGAAGTGTGTGG	–	NR_029593
Let-7a	TGAGGTAGTAGGTTGTATAGTT	–	NR_029725
U6	GCG, CGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT	NR_003027
HIF-α	ACCTTCATCGGAAACTCCAAAG	CTGTTAGGCTGGGAAAAGTTAGG	NM_010431
ER-α	CCTCCCGCCTTCTACAGGT	CACACGGCACAGTAGCGAG	NM_007956
PDCD4	GATTAACTGTGCCAACCAGTCCAAAG	CATCCACCTCCTCCACATCATACAC	NM_001168492
VEGF	TGAACTTTCTGCTCTCTTGGG	GGTTCGCTGGTAGACATCG	–
CS	GGACAATTTTCCAACCAATCTGC	AGTCAATGGCTCCGATACTGC	–
GAPDH	TCAACAGCAACTCCCACTCTTCC	ACCCTGTTGCTGTAGCCGTATTC	NM_008084

ER-α: estrogen receptor alpha, CS: citrate synthase, HIF-1a: hypoxia-inducible factor 1-a, PDCD4: programmed cell death protein 4, TNF-α: tumor necrosis factor alpha, and VEGF: vascular endothelial growth factor.

running at the end of their dark cycle (0700) at gradually increasing speeds (10, 12, 16, and 18 m/min) and 0% inclination. Following the acclimation, the interval exercise training protocol began at 16–18 m/min, 0% grade, 10–14 min, 5 days/week for 5 weeks [25]. The exercise intensity (running speed) gradually increased each week because of strong relationship between treadmill running speed and VO2 from sub maximal intensities up towards maximal levels in mouse and rat [26]. However, weekly increase in running speed during a training period could only be used as a guide to assess adaptation to exercise training, therefore, control of the exercise intensity and the integrated effects of training require regulation of running speed based on serial measurements of VO2max. All animals performed the same intensity and duration in each session. Any electrical stimulation was not used, and the mice were encouraged to run by a gentle tap on the tail or hindquarters. The untrained control animals were put on the switched-off treadmill during the same 5 weeks as the exercise-trained groups. The exercise training protocol was stopped 48 h before sacrifice.

2.7. Body weight and tumor size measurement

Animals were weekly weighed and regularly monitored for abnormal sequels. Tumor volume was measured by a digital vernier caliper (Mitutoyo, Japan) on a weekly basis, and reported as cm^3 using the following formula [27]:

$$V = 1/6(\pi LWD)$$

where L = length, W = width and D = depth.

2.8. Blood and tissue sampling

To avoid acute exercise response, the mice were euthanized 48 h after the last exercise session. During euthanasia period, blood (1.5 ml) was withdrawn intracardially. Animals were finally euthanized using cervical dislocation. Blood samples were then centrifuged for

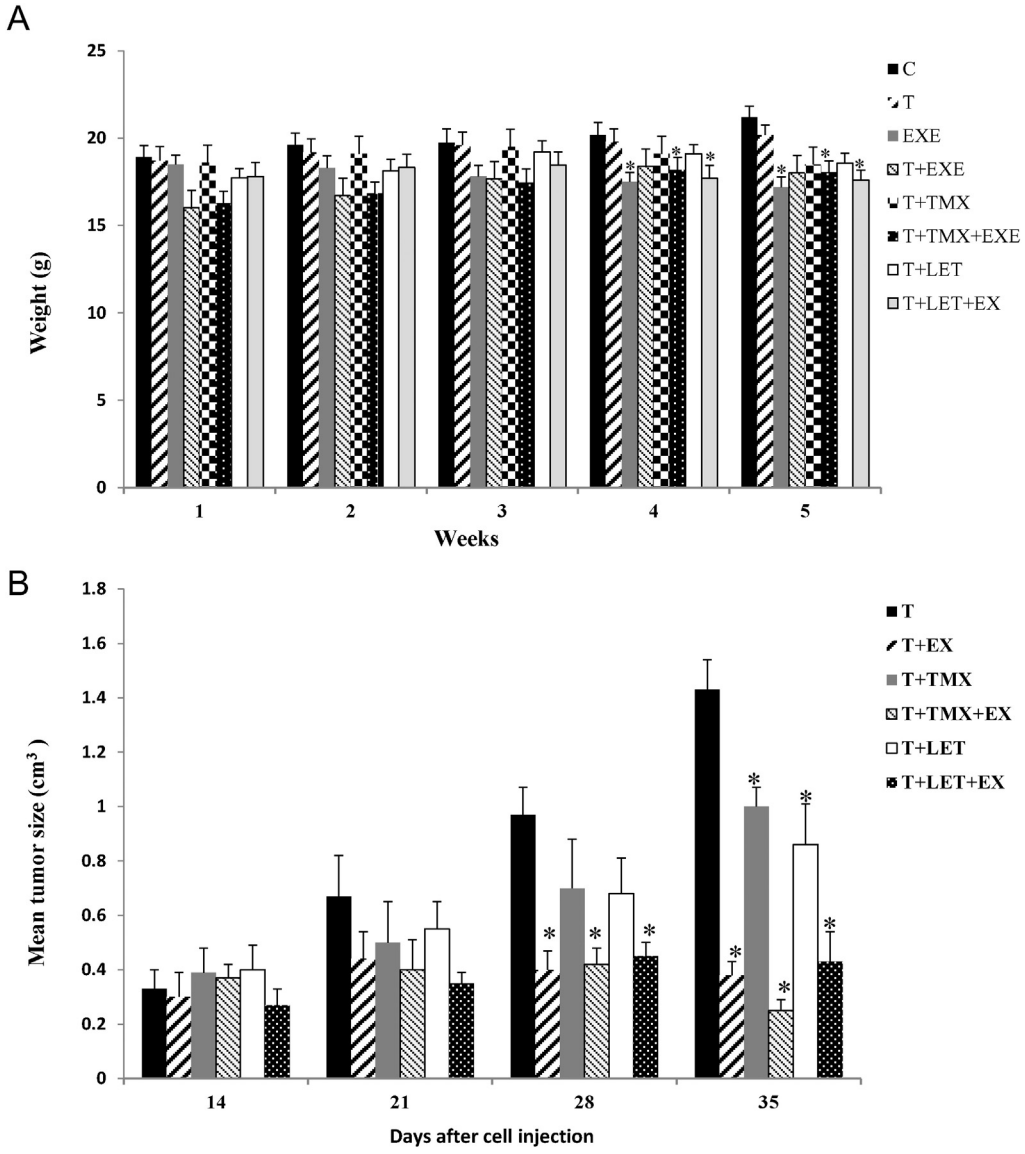


Fig. 1. The effect of interval exercise training, tamoxifen and letrozole on weight (g) (A) and tumor size (cm^3) (B) in mammary tumor-bearing mice. Body weight was weekly measured during the treatment period. The body weight of mice of exercise group was significantly decreased than control group ($P < 0.05$). Interval exercise training also resulted in lower body weight in T + TMX + EX, T + LET + EX and T + EXE groups than T group ($P < 0.05$). The average tumor volume was significantly less in T + EX and T + TMX + EX, T + LET and T + LET + EX groups than T group at 28 and 35 days after tumor cell injection. Tumor volume was measured by a digital vernier caliper on a weekly basis and reported as cm^3 using the following formula: $V = 1/6(\pi LWD)$, where L = length, W = width and D = depth. Data reported are mean ± SD; *$P < 0.05$ compared to T group. T: tumor, T + EX; tumor + exercise, T + TMX; tumor + tamoxifen, T + TMX + EX; tumor + tamoxifen + exercise, T + LET; tumor + letrozole, and T + letrozole + EX; tumor + letrozole + exercise.

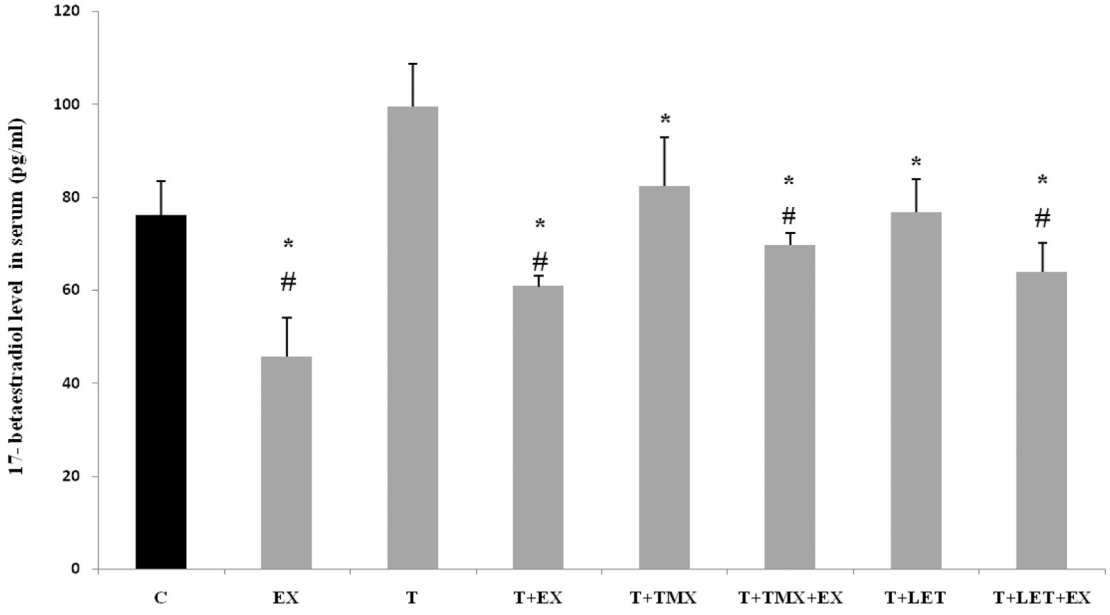


Fig. 2. The exercise training, letrozole and tamoxifen effects on 17-beta estradiol serum level in mice breast cancer. 17-Beta estradiol level was significantly decreased in T + EX, T + TMX, T + TMX + EX, T + LET, and T + LET + EX groups in compared to C and T groups. Data reported are mean ± SD, $^{*}P < 0.05$ compared to control group, and $^{\#}P < 0.05$ compared to tumor group. C; control without any manipulation, EX; with only exercise training manipulation, T; tumor, T + EX; tumor + exercise, T + TMX; tumor + tamoxifen, T + TMX + EX; tumor + tamoxifen + exercise, T + LET; tumor + letrozole, and T + LET + EX; tumor + letrozole + exercise.

10 min at 4000 rpm, and serum was collected and stored at −80 °C for further analysis. Tumor tissues were fixed in 10% formaldehyde, passaged and embedded in paraffin. Paraffin blocks were then sectioned (3 μm) and stained with hematoxylin and eosin (H & E) [27,28].

2.9. IL-10, TNF-α and VEGF levels in tumor tissue

Fresh-frozen tumor tissues (100 mg) were homogenized in 10 volumes of an ice-cold buffer containing 50 mM Tris_HCl (pH 7.8), 2 mM potassium phosphate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 mM leupeptin, 5 mg/ml aprotinin, and 1 mM 4-[(2-aminoethyl) benzenesulfonyl fluoride] using a motor-driven glass pestle. The homogenates were immediately centrifuged at 12,000g for 20 min at 4 °C, and the supernatant was removed as the detergent-soluble fraction. Protein concentrations were determined using the Bio-Rad Protein Assay with BSA for the standard curve. The samples were stored immediately in aliquots at −80 °C for subsequent ELISA analysis. IL-10, TNF-α and VEGF levels were measured in duplicate by using commercially available mouse ELISA kits (Abcam, Germany) according to the manufacturers' instructions.

2.10. Total RNA isolation and cDNA synthesis

Total RNA was extracted from 50 to 100 mg of tumor and soleus muscle tissue with Trizol solution according to the manufacturer's instructions (Invitrogen). cDNA synthesis was performed using Qiagen cDNA synthesis kit (Qiagen, cat: 205311). Briefly 1 μg of RNA was added to the reaction mixture (gDNA Wipeout Buffer, Quantiscript Reverse-Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water) which then was incubated at 42 °C for 15 min. The reverse transcription was terminated at 95 °C for 3 min and samples were stored at −20 °C until used for real-time PCR analysis. cDNA synthesis of microRNAs (1 μg total RNA as input) was carried out according to the kit protocol (Stratagene, USA).

For primer design, the gene and microRNA sequences were extracted from NCBI gene bank (www.NCBI.nlm.nih.gov) and microRNA gene bank (www.mirbase.org), respectively. We used Primer 3 software to design PCR primers, and then submitted them to the selected BLAST databases for checkpoint. The primers were designed for amplification of 88–120 bp by the rotor-Gene Q PCR device. The lyophilized primers were synthesized by Cinnagen Co.

2.11. Evaluation of ER-α, HIF-α and PDCD4 mRNA expression in tumor, and VEGF and citrate synthase in soleus muscle

To determine the mRNA relative expression of ER-α, HIF-α, PDCD4, VEGF and Citrate Synthase (CS), qRT-PCR was performed by using the SYBR Green dye. The thermal cycling program was as follows: 94 °C for 3 min followed by 30 cycles of 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 0.5 min. GAPDH mRNA was used for normalization of the gene expression analysis. The sequence of PCR primers used for the amplification of the protein coding genes in this study has been shown in Table 1.

2.12. Detection of miR-206, miR-21 and Let-7a using Q-RT real-time

High-Specificity miRNA QPCR Core Reagent Kit provides the reagents for quantitative PCR amplification of cDNA templates derived from miRNAs within a total RNA population. Because of their short length, miRNAs are difficult to detect with standard QRT-PCR protocols. As a first step, we used the miRNA 1st-strand cDNA synthesis kit (Stratagene, USA) to elongate miRNAs in a polyadenylation reaction and then reverse transcribed the polyadenylated RNA into QPCR-ready cDNA. The target of interest was then amplified and detected using the high-specificity miRNA QPCR core reagent kit (Stratagene, USA). The universal reverse primer served as the downstream primer in the QPCR reaction, and the specificity of the QPCR reaction was provided by the miRNA-specific forward primer.

2.13. Immunohistochemistry examinations

Immunohistochemistry studies were performed on multiple 3 to 5-μm tissue sections, sequentially each three sections on one slide prepared after 500 μm deeper than the earlier section and totally 30 sections examined, using the avidin-biotin immunoperoxidase method

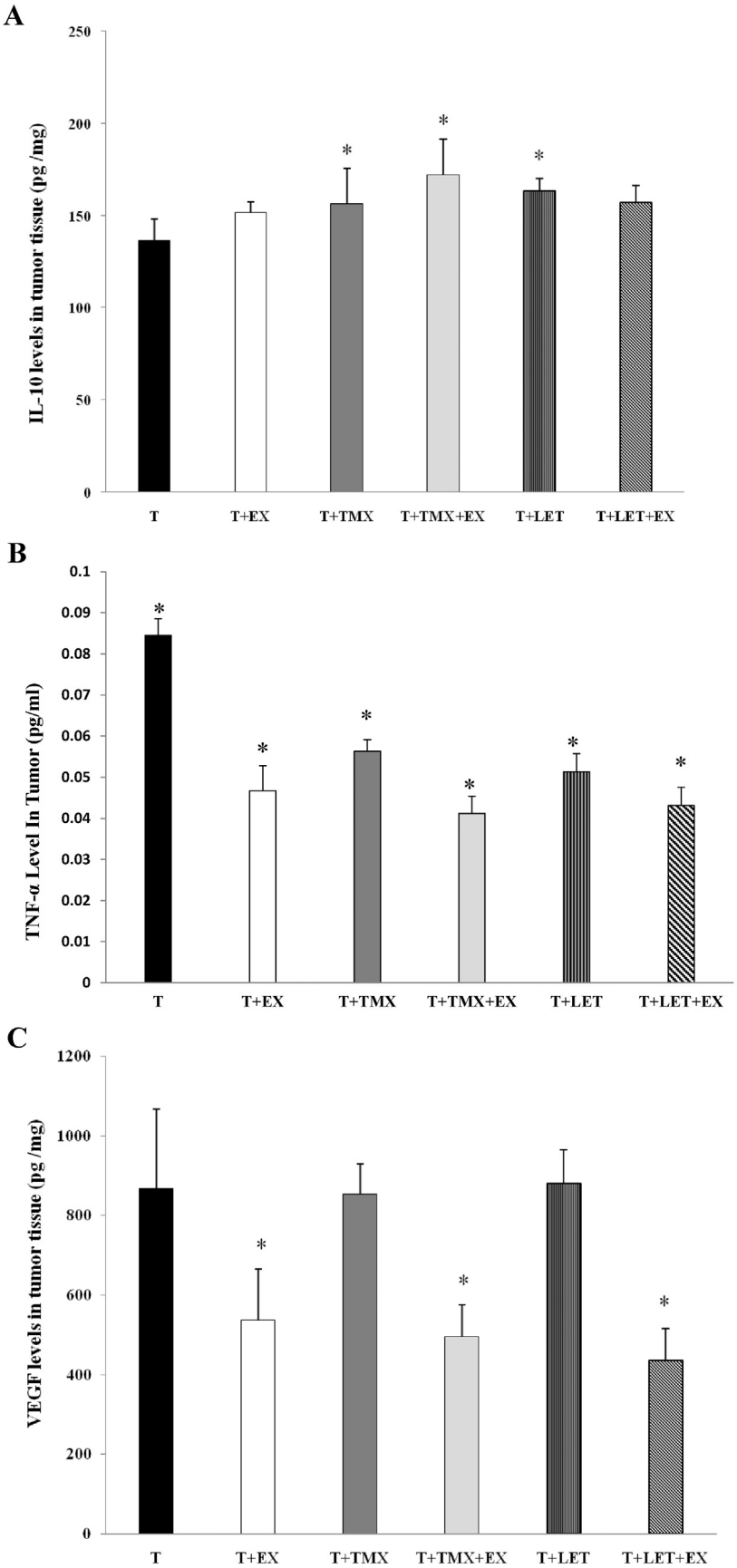


Fig. 3. The exercise training, tamoxifen and letrozole effects on tumor tissue IL-10 (A) and VEGF (B) levels in mice breast cancer. A) IL-10 level was significantly increased in T + TMX, T + TMX + EX and T + LET groups than tumor group. Data reported are mean ± SD; $^{*}P < 0.05$ compared to tumor group. B) VEGF level was significantly decreased in T + EX, T + TMX + EX and T + LET + EX groups than tumor group. Data reported are mean ± SD; $^{*}P < 0.05$ compared to tumor group. T; tumor, T + EX; tumor + exercise, T + TMX; tumor + tamoxifen, T + TMX + EX; tumor + tamoxifen + exercise, T + LET; tumor + letrozole, and T + LET + EX; tumor + letrozole + exercise.

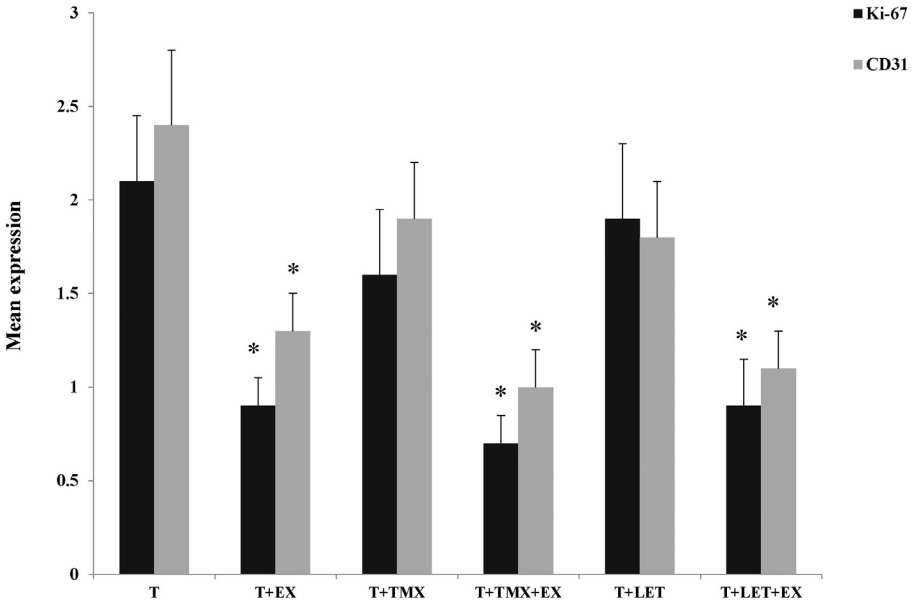


Fig. 4. The exercise training, tamoxifen and letrozole effects on Ki67 and CD31 protein expression by the immunohistochemical assay on mice breast tumor. Ki67 and CD31 protein expression was significantly decreased in T + EX, T + TMX + EX and T + LET + EX groups than T group ($P < 0.05$). Data reported are mean ± SD; *$P < 0.05$ compared to tumor group. T; tumor, T + EX; tumor + exercise, T + TMX; tumor + tamoxifen, T + TMX + EX; tumor + tamoxifen + exercise, T + LET; tumor + letrozole, and T + LET + EX; tumor + letrozole + exercise.

[27]. For the angiogenic and proliferative studies of tumor cells, sections were stained with polyclonal mouse antiRat/Rabbit CD31 and Ki67 antibodies, respectively (DAKO Corporation, USA) according to the manufacturer's instructions. Briefly, the paraffin sections were deparaffinized with Xylene, and rehydrated through a series of descending graded ethanol solutions. The slides were placed in TBS-EDTA buffer and microwaved for 15 min at 90 °C. Endogenous peroxidase activity was blocked by incubation in 0.3% H2O2 buffer for 15 min. The biotinylated secondary antibody and avidin-biotin complex with horseradish peroxidase were applied followed by the addition of chromogen 3,3′-diaminobenzidine (Sigma Chemical). The criteria used to evaluate the angiogenesis of the tumor via CD31 marker was based on the semi-quantitative score as follows [27,29]: 1 vessel/1–3 sections = 0, 2–3 vessels/section = 1, 3–6 vessels/section = 2 and ≤7 vessels/section = 3.

Ki67 positive cells were also identified, and color intensity was semi-quantified using the following protocols [27,29,30]: No staining: 0, faint/barely staining up to 1/3 of cells: 1, moderate staining in 1/3 to 1/2 of cells: 2 and strong staining in more than 1/2 of cells: 3.

2.14. Statistical analysis

All data presented as mean ± SD. Two-way ANOVA was used to asses main effects of exercise training and tamoxifen, and interaction among exercise training, tamoxifen and letrozole for all variables in mice bearing model of breast cancer. If significant effects were found, Bonferroni post hoc test was used to determine the source of the difference. All analysis were performed with statistical analysis was performed using SPSS statistical software (version 13) with the significance level set at $P < 0.05$.

3. Results

3.1. Clinical observation, body weight and tumor size

There were no behavioral changes in the animals during the course of administration or follow up period. Body weight was weekly measured during the treatment period. The body weight in the exercise group had significantly reduced than control group at the end the study ($P < 0.05$) (Fig. 1A). The exercise training resulted to lower body weight in T + TMX + EX, T + LET + EX and T + EXE vs. T group ($P < 0.05$).

The average tumor volume was significantly less in T + EX, T + TMX + EX, T + LET, and T + LET + EX groups than T group at 28, 35 days after the tumor cell injection ($P < 0.05$). Mean final tumor volume reached to approximately 1.43 ± 0.11, 0.38 ± 0.05, 0.1 ± 0.07, 0.25 ± 0.04, 0.86 ± 0.15, and 0.43 ± 0.13 cm^3 in T, T + EX, T + TMX, T + TMX + EX, T + LET, and T + LET + EX groups, respectively (Fig. 1B). There were no signs of tumor metastasis in the main organs of both treatment and control groups.

3.2. 17-Beta estradiol levels in serum

Serum E2 level was significantly lowered in T + EX (60.77 ± 9.31 pg/ml) group than others ($P < 0.05$) (Fig. 2). The results showed that serum E2 level in EX group (45.72 ± 7.12 pg/ml) was significantly lower than T group (99.41 ± 8.39 pg/ml) ($P < 0.05$) (Fig. 2). In addition, it was significantly decreased in T + TMX + EX, T + TMX, T + LET and T + LET + EX groups in comparison with T group ($P < 0.05$) (Fig. 2).

3.3. Tumor IL-10, TNF-α and VEGF levels

We measured IL-10 level because of its effect on angiogenesis. It can inhibit cancer growth by hampering tumor angiogenesis and invasiveness. Tumor IL-10 level was higher in T + TMX + EX group (171.917 ± 19.53 pg/mg) than other groups. Its level was significantly increased in T + TMX (171.917 ± 19.34 pg/mg) and T + LET (163.44 ± 19.53 pg/mg) groups as compared to T group (136 ± 11.8 pg/mg) ($P < 0.05$). However, IL-10 level in T + EX and T + LET + EX groups was higher than T group, but this increase was not statistically significant (Fig. 3A). TNF-α as pro-inflammatory cytokine was significantly decreased in T + EX (0.0467 ± 0.004 pg/mg), T + TMX (0.0563 ± 0.0061 pg/mg), T + TMX + EX (0.041 ± 0.003 pg/mg), T + LET (0.05 ± 0.004 pg/mg) and T + LET + EX (0.04 ± 0.005 pg/mg) groups compared to T group (0.085 ± 0.004 pg/mg) ($P < 0.05$) (Fig. 3B). Tumor VEGF level was significantly

decreased in T + EX (537 ± 127 pg/mg) group as compared to T group (867 ± 199 pg/mg) ($P < 0.05$), and it was also more decreased in T + TMX + EX (496 ± 75 pg/mg) and T + LET + EX (436.25 ± 436.25 pg/mg) ($P < 0.05$) (Fig. 3C).

3.4. Angiogenesis and proliferation examinations

To further study the mechanism of the effects of the exercise training, tamoxifen and letrozole on angiogenesis and proliferation, the immunohistochemical assay revealed that Ki67 protein expression was significantly decreased in T + EX (0.9 ± 0.35), T + TMX + EX (0.7 ± 0.15) and T + LET + EX (0.9 ± 0.25) groups than T (2.1 ± 0.35) group ($P < 0.05$) (Fig. 4). Also, CD31 protein expression was significantly decreased in T + EX (1.3 ± 0.2), T + TMX + EX (1.00 ± 0.3) and T + LET + EX (1.1 ± 0.2) groups than T (2.4 ± 0.4) group ($P < 0.05$) (Fig. 4).

3.5. miR-206, miR-21 and let-7a

The results of this study indicate that miR-206 was over-expressed in T + EX, T + LET and T + LET + EX groups than T group (Fig. 5A). Moreover, miR-206 over-expression in T + EX group (10.58 ± 2.93 fold) was more than T + LET + EX group (5.91 ± 1.67 fold) ($P < 0.05$) (Fig. 5A). It was also up-regulated in T + TMX (5.91 ± 2.65 fold) and T + TMX + EX (9.51 ± 1.86 fold). On the other hand, miR-21 expression as oncogene micro-RNA was significantly down-expressed in T + LET + EX group (0.154 ± 0.06 fold) than T group ($P < 0.05$) (Fig. 5B). MiR-21 expression was decreased in T + EX (0.64 ± 0.032 fold), T + TMX (0.29 ± 0.021 fold) and T + TMX + EX (0.056 ± 0.0023 fold) groups more than T group ($P < 0.05$) (Fig. 5B). Let-7a over-expressed in T + EX (7.66 ± 1.1 fold), T + TMX (6.97 ± 1.25 fold), T + LET (1.96 ± 0.81 fold), T + TMX + EX (7.21 ± 1.03 fold), T + LET + EX (6.93 ± 1.3 fold) groups than T group ($P < 0.05$) (Fig. 5C).

3.6. Tumor ERα, HIF-α and PDCD4 mRNA expression

ERα mRNA expression was decreased in T + EX (0.042 ± 0.01 fold) and T + LET + EX (0.22 ± 0.15 fold) groups in comparison with T group ($P < 0.05$). It was not significantly lower in T + LET (0.838 ± 0.21 fold) than other groups (Fig. 6A). In T + TAM (0.051 ± 0.032 fold) and T + TAM + EX (0.47 ± 0.013 fold) groups, ERα mRNA expression was significantly decreased compared to T group (Fig. 6A).

HIF-α mRNA expression was decreased in T + EX (0.22 ± 0.01 fold) and T + LET + EX (0.55 ± 0.12 fold) groups in comparison with T group ($P < 0.05$). It was decreased in T + TMX group (0.09 ± 0.012 fold) than other groups (Fig. 6B). PDCD-4 mRNA expression was increased in T + EX (3.957 ± 1.36), T + TMX (2.65 ± 0.82), T + LET (2.83 ± 0.71 fold), T + TMX + EX (4.87 ± 1.03 fold), T + LET + EX (6.77 ± 3.1 fold) groups than T group ($P < 0.05$) (Fig. 6C).

3.7. Citrate synthase and VEGF mRNA expression in soleus muscle

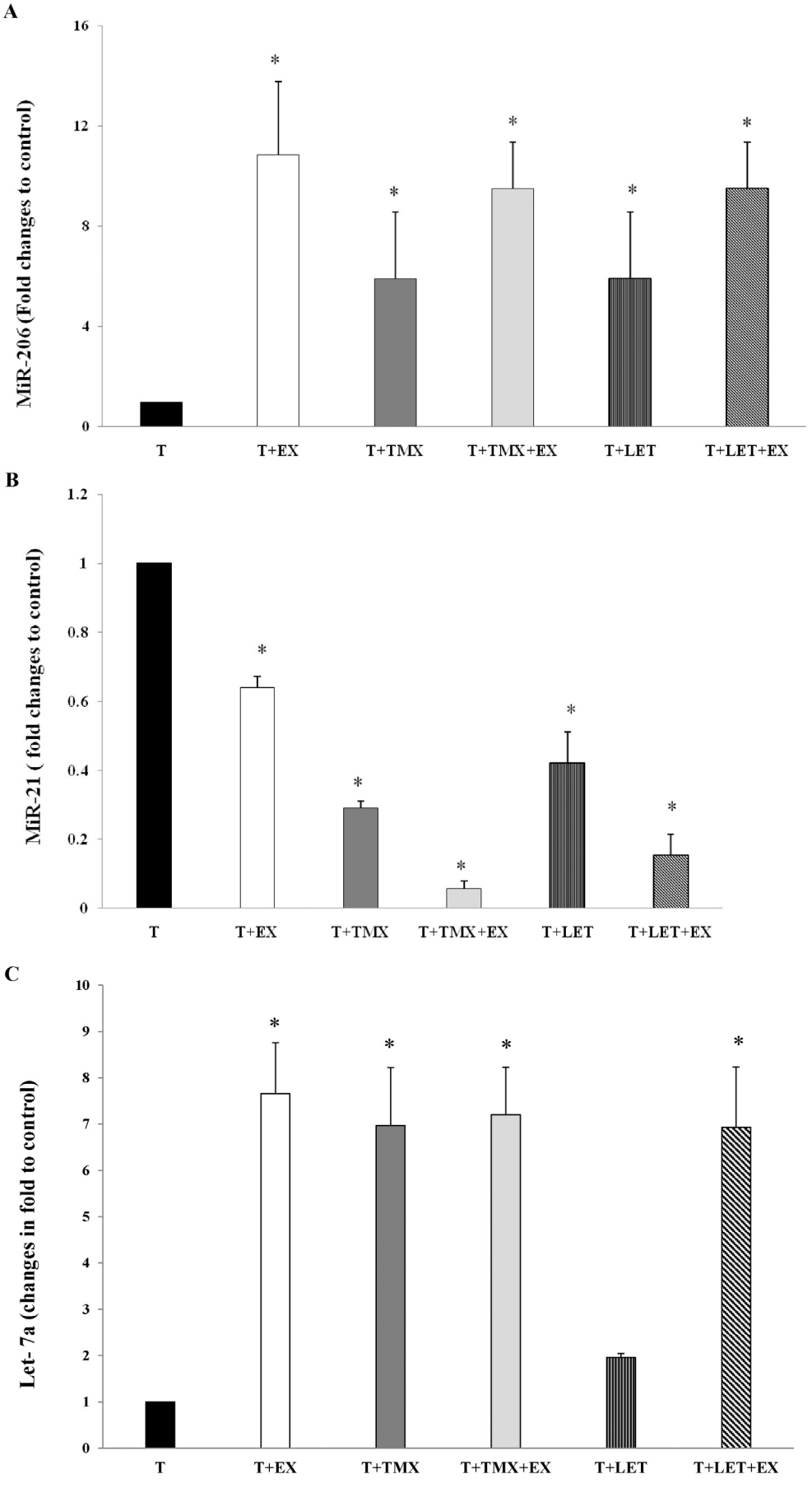
Our results indicated that the exercise training induced the adaptation in soleus muscle by increasing citrate synthase (CS) and VEGF expression. In contrast to tumor tissue, CS mRNA expression in soleus muscle was significantly increased in healthy animals (EX) (6.29 ± 1.28 fold) and T + EX (8.53 ± 1.47 fold) than T group after 5 weeks of interval exercise training ($P < 0.05$) (Fig. 7A). Also, CS mRNA expression was increased in the healthy animals (1.58 ± 0.38 fold) and T + EX (1.83 ± 0.79 fold) than T group after 5 weeks of interval exercise training ($P < 0.05$) (Fig. 7B).

4. Discussion

The main findings of this study showed that the interval exercise training in combination with tamoxifen and letrozole decreased tumor growth, which associated with the decreased expression of ERα, HIF-α, VEGF and miR-21, and the increased expression of let-7a, miR-206 and IL-10. The expression of CD31 and Ki67 proteins, as angiogenesis and proliferation markers, was decreased in the treated mice in compared to control group. TNF-α level in tumor tissue was decreased after the interval exercise training and hormone therapy. Therefore, it seems that miR-21, miR-206 and let-7a pathways may involve in anti-angiogenesis effects of the interval exercise training with hormone therapy in mice breast tumor (Fig. 8).

Exercise training is now considered as an important adjuvant therapy to reduce cancer symptoms and to improve the pharmaceutical therapies [31]. It is now crucial to find the safe and the effective lifestyle modifications, including the physical activity for decreasing breast cancer development. Our results indicated that miR-21 was down-regulated after 5-weeks of the interval exercise training and the hormone therapy. Liu et al. found that overexpression of miR-21 could up-regulate the expression of HIF-1α and VEGF, and induced tumor angiogenesis [10]. VEGF and estrogen expression were studied extensively yielding, however, the results are controversial. For example, VEGF expression in breast cancer cells (MCF7) was increased when incubated with estrogen, as well as with tamoxifen [32]. On the other hand, tamoxifen in combination with estradiol could decrease extracellular VEGF in solid breast tumors as compared with estradiol treatment alone [33]. Additionally, several studies showed the increased expression of VEGF by hypoxia and up-regulation of HIF-1 that induced the formation of new vessels [34]. In this regard, down-regulation of HIF-1α after the exercise training can suppress this function. In our study, the interval exercise training plus tamoxifen down-regulated more the expression of HIF-1α and ER-α than the exercise training plus letrozole. In addition, tamoxifen reduced VEGF and HIF-1α expression. Thus, according these results the exercise training may reduce tumor angiogenesis by affecting HIF-1α and ER-α pathway [35]. On the other hand, we did not find the significant changes in VEGF and HIF-1α expression by letrozole administration but the interval exercise training plus letrozole has significant effects. Therefore, it may be explained the down-regulatory effects of tamoxifen and the exercise training on HIF-1α in opposite to letrozole only administration. In contrast to tumor samples, VEGF mRNA expression was significantly decreased in the soleus muscle of the trained animals. HIF-1α acts as a regulator for expression of the genes involved in the hypoxia response of most mammalian cells [36]. There are some documents that the exercise training through HIF-1α can induce VEGF expression via increased angiogenesis in the skeletal muscle [37,38]. On the other hand, we demonstrated the decreased expression of HIF-1α in tumor tissues after the exercise training. Therefore, the observed differences between VEGF expression in the tumor tissue and the soleus muscle may be explained by the decreased expression of HIF-α in tumor tissues of the trained animals.

Fig. 5. The exercise training, tamoxifen and letrozole effects on expression of miR-206 (A), miR-21 (B) and Let-7a (C) in mice breast tumor. A) Relative miR-206 levels were determined by qReal-time PCR, expressed as fold change after normalization to U6. Its expression was significantly increased in T + EX, T + TMX, T + TMX + EX, T + LET, and T + LET + EX groups than T group ($P < 0.05$). B) Relative miR-21 levels were determined by qReal-time PCR, expressed as fold change after normalization to U6. Its expression was significantly decreased in T + EX, T + TMX, T + TMX + EX, T + LET, and T + LET + EX groups than T group ($P < 0.05$). C) Relative Let-7a levels were determined by qReal-time PCR, expressed as fold change after normalization to U6. Its expression was significantly increased in T + EX, T + TMX, T + TMX + EX and T + LET + EX groups than T group ($P < 0.05$). Data reported are mean ± SD; *$P < 0.05$ compared to tumor group. T; tumor, T + EX; tumor + exercise, T + TMX; tumor + tamoxifen, T + TMX + EX; tumor + tamoxifen + exercise, T + LET; tumor + letrozole, and T + LET + EX; tumor + letrozole + exercise.


A
MiR-206 (Fold changes to control)
16
12
8
4
0
T
T+EX
T+TMX
T+TMX+EX
T+LET
T+LET+EX
B
MiR-21 (fold changes to control)
1.2
1
0.8
0.6
0.4
0.2
0
T
T+EX
T+TMX
T+TMX+EX
T+LET
T+LET+EX
C
Let- 7a (changes in fold to control)
10
9
8
7
6
5
4
3
2
1
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T
T+EX
T+TMX
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T+LET
T+LET+EX

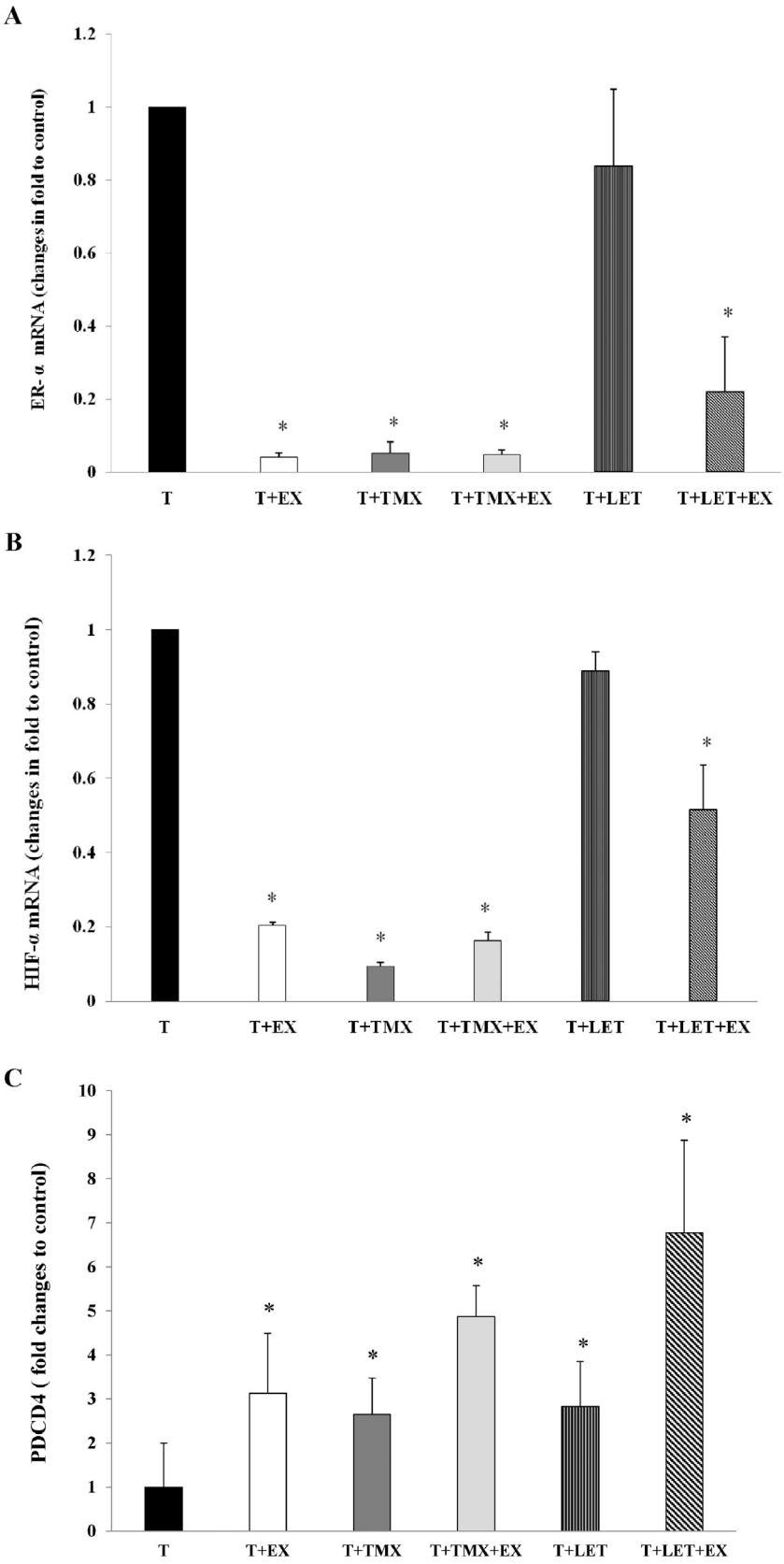

Fig. 6. The exercise training, tamoxifen and letrozole effects on mRNA expression of ERα (A), HIF-α (B) and PDCD4 (C) in mice breast tumor. A) Relative ERα mRNA level was determined by qReal-time PCR, expressed as fold change after normalization to GAPDH. Its expression was significantly decreased in T + EX, T + TMX, T + TMX + EX, and T + LET + EX groups than T group ($P < 0.05$). B) Relative HIF-α mRNA level was determined by qReal-time PCR, expressed as fold change after normalization to GAPDH. Its expression was significantly decreased in T + EX, T + TMX, T + TMX + EX, and T + LET + EX groups than T group ($P < 0.05$). C) Relative PDCD4 mRNA level was determined by qReal-time PCR, expressed as fold change after normalization to GAPDH. Its expression was significantly decreased in T + EX, T + TMX, T + TMX + EX, and T + LET + EX groups than T group ($P < 0.05$). Data reported are mean ± SD; *$P < 0.05$ compared to tumor group. T; tumor, T + EX; tumor + exercise, T + TMX; tumor + tamoxifen, T + TMX + EX; tumor + tamoxifen + exercise, T + LET; tumor + letrozole, and T + LET + EX; tumor + letrozole + exercise.

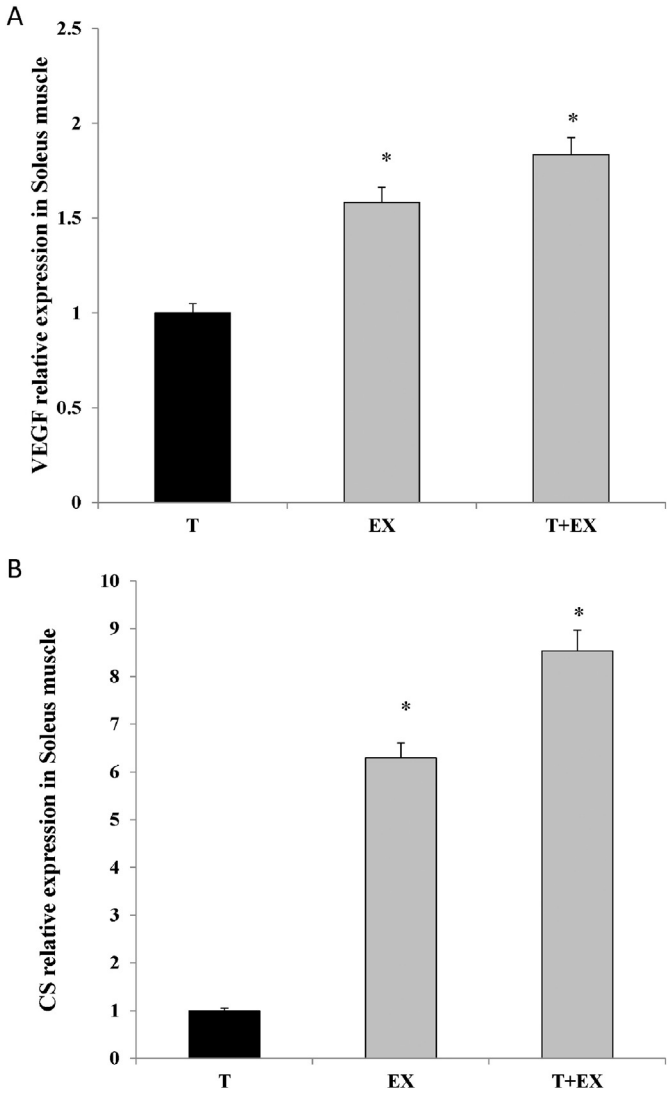

Fig. 7. Relative CS (A) and VEGF (B) mRNA expression in soleus muscle was determined by qReal-time PCR, expressed as fold change after normalization to GAPDH. A) Citrate synthase mRNA expression in soleus muscle was increased significantly in healthy animals (EX) and T + EX than T group after 5 weeks of interval exercise training ($P < 0.05$). B) VEGF mRNA expression was increased in soleus muscle of healthy animals (EX) and T + EX than T group after 5 weeks of interval exercise training ($P < 0.05$). Data reported are mean ± SD; *$P < 0.05$ compared to tumor group. Citrate synthase (CS), T; tumor, T + EX; tumor + exercise, EX; exercise.

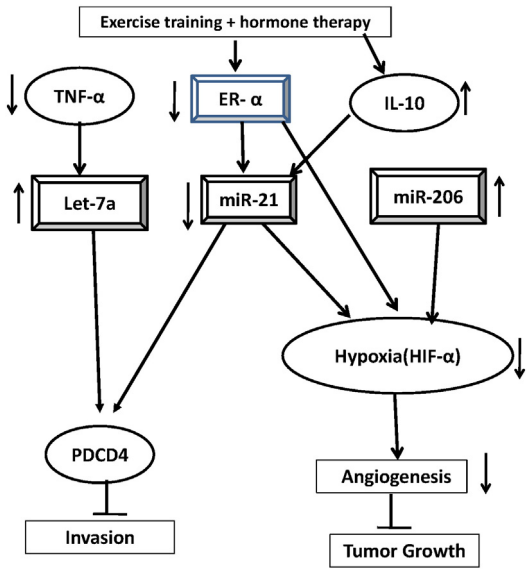

Fig. 8. Schematic pathway of anti- angiogenic effects of interval exercise training and hormone therapy in mammary tumor-bearing mice.

Estrogens stimulate angiogenesis through different processes. In xenograft experiments, estrogens stimulate angiogenesis of implanted breast tumors, and anti-estrogens inhibit angiogenesis [37]. It was also found that tamoxifen reduces the vascular density within the tumor [44], thereby causing tumor regression. Estrogen signaling pathway plays a central role through ER-α in breast cancer. Also, inhibition of ER-α activity has proven an effective treatment option in breast and endometrial cancers [38]. In a study with ER- α knockout mice, loss of ER-α prevented E2- stimulated angiogenesis [39]. Antitumor effect of tamoxifen may in fact relate to an anti-angiogenic action of this estrogen receptor agonist/antagonist [37]. Therefore, we speculate that the anti-angiogenic effects of the exercise training and tamoxifen may partly mediated by decreased expression of ER-α in mice breast tumors.

We also showed that IL-10 level in the tumor tissues was decreased after 5 weeks of the interval exercise training and hormone therapy. IL-10 can inhibit tumor-induced angiogenesis and enhance the production of tumor-toxic molecules (e.g., nitric oxide), which can lead to tumor regression [20,39]. Endurance exercise training can increase IL-10 protein and IL-10/TNF-α ratio in rat adipose and muscle tissues [40]. IL-10 as an anti-inflammatory cytokine can inhibit angiogenesis, which is associated with down-regulation of VEGF expression. This effect can be mediated by ischemia and hypoxia as well as our data that are the primary event leading to a local inflammatory reaction and angiogenesis [41].

We also observed that miR-206 expression was higher in the exercise training and hormone handling. A recent study showed that miR-206 transfection can significantly down-regulate VEGF expression in the lung cancer cells [12]. On the other hand, it seems that miR-206 may be one of several factors that can inhibit ERα expression at the posttranscriptional level, leading to loss of both ERα mRNA and protein [42]. Adams et al. showed miR-206 expression was strongly inhibited by ERα agonists [11]. Additionally, they suggested that miR-206 affects the estrogen signaling by targeting mRNAs encoding ERα-associated co-regulatory proteins. And elevated miR-206 levels in MCF-7 cells can ultimately result in the reduced cell proliferation, enhanced apoptosis, and reduced expression of multiple estrogen-responsive genes [43,44].

Let-7 family has important role in regulating cell proliferation and differentiation [45]. There are some evidence that let-7a is down-regulated in breast cancer [46]. Iorio et al. [47] observed that expression of various let-7 miRNAs was down-regulated in breast cancer samples with either lymph node metastasis or higher proliferation index. According these results they suggested that a reduced let-7 expression could be associated with a poor prognosis [47]. We also observed let-7a expression decreased in tumor tissue after exercise training and tamoxifen.

CD31 expression was also decreased after the exercise training as well as hormone therapy together exercise training in our study. Zielinski et al. demonstrated that exercise training decreased blood vessel density in subcutaneous lymphoid tumors [48]. In contrast, Jones et al. showed that the voluntary wheel running increased the number of the perfused tumor vessels [49]. Kim et al. [50] also investigated the effects of the high fat diet on breast tumor growth and cancer metastasis. They showed that increased expression of CD31 and Ki67 associated with higher tumor volume and high fat diet. They also showed that the dietary fat not only increased the expression of CD31 and VEGF, but also increased the expression of CD68 and CD45 in tumor tissues, indicating an increase in the immune cell infiltration and angiogenesis [50,51]. Ferrandina et al. revealed that tamoxifen was the effective in inhibiting the proliferative factor such as Ki67 [52]. HIF-1α has been correlated to

markers of the aggressive breast cancer cells such as Ki67 and ERα [16]. Therefore, decreased CD31 and Ki67 expression can indicate that the density of tumor blood vessels is decreased as a result of the interval exercise training and hormone therapy in the present study.

In summary, the combination hormone therapy with the exercise training could down-regulate the expression of ERα, miR-21, HIF-1α, CD31, Ki67 and VEGF, and up-regulate the expression of miR-206, let-7 and IL-10 that led to reducing the angiogenesis and tumor growth. Therefore, miR-21, miR-206 and let-7a pathways may involve in anti-angiogenesis effects of the interval exercise training with hormone therapy in mice breast tumor.

Declaration of interest

The author(s) report no conflicts of interest and are responsible for the content of the paper.

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