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ORIGINAL ARTICLE

The impacts of an eight-week moderate aerobic exercise training on some gene expression involved in cholesterol metabolism in ovariectomized rats

Maryam Emamian Rostami[1] · **Rozita Fathi**[1,2] · **Khadijeh Nasiri**[1]

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Abstract

Background Estrogen depletion in postmenopausal women and animal models of ovariectomy is associated with some undesirable alternations in lipid metabolism, the adverse impacts of which could be improved through regular exercise training; however, molecular mechanisms underlying this process are not fully understood. In this study, the impacts of an 8-week moderate aerobic exercise training on plasma lipid profile, liver enzymes, and some gene expression involved in cholesterol metabolism were investigated in ovariectomized rats.

Methods Forty female Wistar rats were randomly divided into four groups including sham-control, exercise training, ovariectomized-control (OVX), and ovariectomized + Exercise training (OVX + E). Three weeks after ovariectomy, the animals began their training on the treadmill (25 m/min, 5 sessions/week) for 8 weeks. The hepatic expression of Fansoid X receptor (FXR), Cholesterol-1-alphahydroxylase 1 (CYP7A1), and Small heterodimeric protein (SHP) along with lipid profile and liver enzymes were assessed.

Result The hepatic expression of FXR, CYP7A1 and SHP genes were down-regulated in OVX rats compared to the exercise group. The levels of triglyceride (TG) and total cholesterol (TC) were significantly increased in OVX and OVX + E rats in comparison to sham and exercise groups. The levels of liver enzymes were also increased in OVX rats. However, exercise did not alter liver enzymes, despite a decrease in total cholesterol in OVX rats.

Conclusion Although ovariectomy could down-regulate the hepatic gene expressions involved in cholesterol metabolism, our exercise protocol could not alter the expression of these genes in OVX rats. These effects may occur due to other variables such as some regulatory mechanisms which are not the subject of the present research.

Keywords FXR · CYP7A1 · SHP · Cholesterol metabolism · Ovariectomy · Exercise training

Abbreviations

ET	Exercise training
TG	Triglycerides
TC	Total cholesterol
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
ALT	Alanine aminotransferase
AST	Aminotransferase
FXR	Farnesoid-X-receptor
SHP	Small heterodimer partner
CYP7A1	Cholesterol 7 alpha-hydroxylase
OVX	Ovariectomy

✉ Rozita Fathi
r.fathi@umz.ac.ir

[1] Department of Exercise Physiology, Faculty of Sport Science, University of Mazandaran, Babolsar, Iran

[2] Athletic Performance and Health Research Center, University of Mazandaran, Babolsar, Iran

Introduction

Women spend more than a third of their life in menopause [1]. Menopause refers to the permanent cessation of menstruation and fertility. Menopause occurs due to an increase in follicle-stimulating hormone (FSH) and a severe decrease in estrogen levels [2]. The onset of menopause in women is significantly associated with the risk of metabolic disorders such as obesity, metabolic syndrome, diabetes, fatty liver, and subsequent cardiovascular diseases [3]. Numerous reports have shown that estrogen deficiency disrupts lipid metabolisms such as an increase in total cholesterol and

low-density cholesterol (LDL) levels and a decrease in bile acid production, which could be recognized as one of the risk factors in these disorders [4]. Although estrogen therapy has been able to moderate these effects to some extent, there is some evidence that proves the estrogen therapy causes hypertriglyceridemia [5, 6].

Bile acids as the amphipathic steroids are responsible for the elimination of excess cholesterol from the liver [7]. Therefore, in this process cholesterol excretion from the peripheral tissue and bloodstream by hepatobiliary and non-hepatobiliary pathway impairment at estrogen deficiency state might also happen [8–10]. Clinical studies demonstrate that postmenopausal women are faced twice as likely as men to develop cholesterol gallstones, defects in bile acids and very low-density cholesterol (VLDL) synthesis, which are known as the main processes of cholesterol removal. Hence, a low plasma level of estrogen causes the accumulation of cholesterol in the liver [11]. In addition, findings from the ovariectomized animal models revealed that estrogen elimination causes to disrupt cholesterol metabolism and leads to an accumulation of cholesterol in the liver [12, 13]. Thus, improving cholesterol metabolism plays an important role in modulating these pathophysiological statuses [14]. Estrogen therapy is most often used to treat common menopausal symptoms and to improve such conditions, however, it is not an effective method to treat post-menopausal complications due to its limitations and side effects [15].

Strong evidence suggests that exercise plays a critical role in inhibiting the cholesterol and bile acid accumulations in the liver and their associated complications, through modulating the gene expression involved in the cholesterol biosynthesis and its excretion [15–19]. Pighon et al. [20] reported that continuous running on a treadmill for 5 weeks acts similarly to estrogen supplementation in the liver; therefore fat accumulation and its metabolic consequence in ovariectomized rat and exercise training can improve bile acid biosynthesis and cholesterol excretion [20]. In the liver a way that is known to regulate cholesterol to bile acids is FXR/ SHP/CYP7A1 pathway; and it also plays a crucial role in cholesterol and bile acid metabolism of the liver [21–23].

Farnesoid X Receptor (FXR) is a member of the family of nuclear receptors that are expressed in the liver, intestine, kidney, adipose, and heart tissue [22]. These receptors can be activated by bile acids [24]. FXR is a bile acid sensor that regulates glucose and lipid metabolism [25]. Bile acids suppress the activity of CYP7A1 by activating FXR in the liver, and they also induce the expression of the SHP gene which inhibits bile acid biosynthesis [22]. SHP like FXR regulates the hepatic bile acid, glucose, and lipid metabolism [26]. On the other hand, it has been reported that there is a positive association between estrogen and the FXR/SHP pathway leading to improve cholesterol and bile acid metabolisms [27]. Moreover, it was reported that exercise training with moderate intensity has estrogenic effects on the gene expression involved in lipid metabolism in the liver [22, 23]. Evidence also showed that exercise training could play an important role in the regulation of cholesterol metabolism in both healthy and OVX rats. It also revealed that physical activity can be more effective than estrogen therapy for improving the serum lipid profile and some metabolic diseases after menopause [15, 28]. Moderate aerobic exercise is also reported to be the most effective and safe method to reduce visceral fat and decrease fatty liver development [29, 30]. Moderate intensity training may exert positive immunomodulation of systemic functions and inflammation [31]. Also, it may be effective to prevent atherosclerosis [32] and reduce the risk of type 2 diabetes [33]. Therefore, regarding the effective role of exercise as a non-pharmacological strategy in improving estrogen depletion in ovariectomized animals, in this study, the researcher evaluated the effect of exercise training on some gene expression involved in cholesterol metabolism (FXR/SHP/CYP7A1) in healthy and ovariectomized rats.

Methods

Animal

All procedures were conducted in accordance with the consent and the "Guiding Principles for the Care and Use of Research Animals" approved by the University of Mazandaran (Iran). Forty adult female Wistar rats (220 ± 10 g) were purchased from Pastor Institute of Iran. Animals (six rats per cage) were housed in an air-conditioned environment (20 ± 2 ℃) under controlled lighting (12 h light-12 h darkness). Animals were allowed to have free access to food and water.

Experimental design

To investigate the effect of aerobic training on the plasma lipid profile, liver enzymes (AST and ALT), and relative expression of FXR, SHP and CYP7A1 genes in the liver, rats were divided in four groups including sham-control, exercise training, ovariectomized-control(OVX), ovariectomized + Exercise training(OVX + E).

Ovariectomy

The rats were anesthetized with ketamine (70 mg/kg) and xylazine (3–5 mg/kg) by intraperitoneal injection and a minor abdominal operation was done. Then, the ovaries were removed in rats of OVX groups. The skin and muscle walls were sutured by a silk thread. In the sham-control group to eliminate the potential effect of the surgical stress, the

anesthesia and surgery steps were performed exactly like the ovariectomized groups without ovarian removal [34].

Exercise training protocol

Exercise training was performed 3 weeks after the ovaries were removed from the rats in OVX groups. During the first week, the animals were accustomed to running on a treadmill without an incline for 12 m/min per 15 min/day. After that, rats ran on a treadmill for eight weeks (25 m/min for 60 min/day). In addition, 5 minutes for warming and cooling were considered for all rats. To equalize the conditions for both experimental and control groups, the control group also walked on a treadmill 10 min a day, for two or three sessions per week at a speed of 10 m/min. During this time, it was attempted to keep the environmental conditions for the control group quite similar to those of the training group [23, 35]. Exercise training was carried out every morning.

Blood and tissue sampling

At the end of period, 48 h after the last training session, animals were sacrificed under anesthesia (ketamine 70 mg/kg and xylazine 3–5 mg/kg) after 12hrs fasting. Blood samples were collected by the abdominal vena cava in tubes containing anticoagulant and were then centrifuged (3000 rpm; 4 °C; 15 min). The plasma was kept in − 80 °C for later assays. Lipid profile including total cholesterol (TC), triglyceride, low-density lipoproteins (LDL), and high-density lipoproteins (HDL), and the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using Hitachi-917 Autoanalyser with the corresponding reagent kit. After blood sampling, livers were quickly removed and immediately rinsed in ice saline and kept in − 80 °C to assay hepatic gene expression.

RNA isolation and quantitative real-time (RT) polymerase chain reaction (PCR)

In order to extract total RNA from rats' liver tissues, RNeasy mini kit (Qiagen, Germany) was used and RNA extraction was performed according to the manufacturer's instructions. Purity and quantity of extracted RNA (260/280 and 260/230 ratios) were determined with a ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription was also carried out with QuantiNova Reverse Transcription Kit (Qiagen, Germany) according to the company's operating instructions. Before the reverse transcription, genomic DNAs were removed from the samples. The RNA (5 µg) was reverse transcribed at 42 °C for 10 min using a combination of oligo-dT and random primers. Primer pairs for three different genes involved in cholesterol metabolism (FXR, SHP and CYP7A1) and one candidate reference gene (β-Actin) were designed using Primer premier version 5 software. The primers were synthesized by Macrogen Company (South Korea). Oligonucleotide primers used for quantitative real-time polymerase chain reaction is shown in Table 1.

Real-time PCR was performed in Rotor gene Corbett 6000 using SYBRGreen method in a total reaction volume of 15 µL. The reaction mixture consisted of 2 µL cDNA (50 ng/µL), 7.5 µL QuantiNovaTM SYBR Green PCR (Qiagen, Germany), 0.2 µL of each primer (10 pmol), and 5.1 µL of ribonuclease-free water. The PCR amplification profile was as follows: 95 °C for 5 min followed by 40 cycles of denaturation at 95 ºC/30 s, annealing temperature at 58 °C for binding of FXR and Beta-Actin primers, and 60 °C for binding of the CYP7A1 and SHP gene primers /35 s, and extension at 72 ºC/20 s. A melting curve analysis was done immediately after the qPCR analysis. The expression levels of the target genes were also measured through $2^{-\Delta\Delta CT}$ method.

Table 1 Oligonucleotide primers used for the quantitative real-time polymerase chain reaction

Gene	Primer Sequence	Accession n Gene	Product Length (Pair)
FXR	Forward 5′ ATCTACCAGCCCGAGAACCC 3′ Reverse 5′ ATTCACCCTCCAAGACATCAGC 3′	NM_021745.1	111
SHP	Forward 5′ CTTTGAGGTGGCCGAGGCT 3′ Reverse 5′ GAGGGTTGTGGTGGGTCTGG 3′	NM_057133.1	108
CYP7A1	Forward 5′ GTGTGACGTTGACATCCGTAAAGAC 3′ Reverse 5′ TGCTAGGAGCCAGGGCAGTAAT3′	X17595.1	119
β–Actin	Forward 5′ CCAAGACGCACCTCGCTATTC 3′ Reverse 5′ CTCACTTCTTCAGAGGCTGCTTTC 3′	NM_031144.3	121

Statistical analysis

In this study, all values are expressed as means and particularly at ± standard error of mean. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Kolmogorov–Smirnov and Levene's test were used to investigate the normal distribution of data and variance homogeneity. Data were analyzed by two-way analysis of variance (ANOVA) and a posthoc multiple comparisons Tukey test was used to compare significant differences between groups. Statistical significance was set at $p < 0.05$.

Results

The results demonstrated that ovariectomy could significantly increase the mean of final body weight in OVX and OVX + E groups, compared to sham-control group ($p < 0.01$, Fig. 1). No significant difference was found between OVX and OVX + E groups for final body weight; and the results also showed that exercise training has not decreased final body weight significantly compared to other groups ($p > 0.01$, Fig. 1).

The mean ± SEM of lipid profile levels in experimental groups are presented in Table 2. The results indicated that the level of TG were significantly increased in OVX group compared to sham and exercise training groups ($p < 0.01$, Table 2). TC levels were significantly increased in OVX group compared to sham-control and exercise training groups ($p < 0.0001$, Table 2). TG levels were also

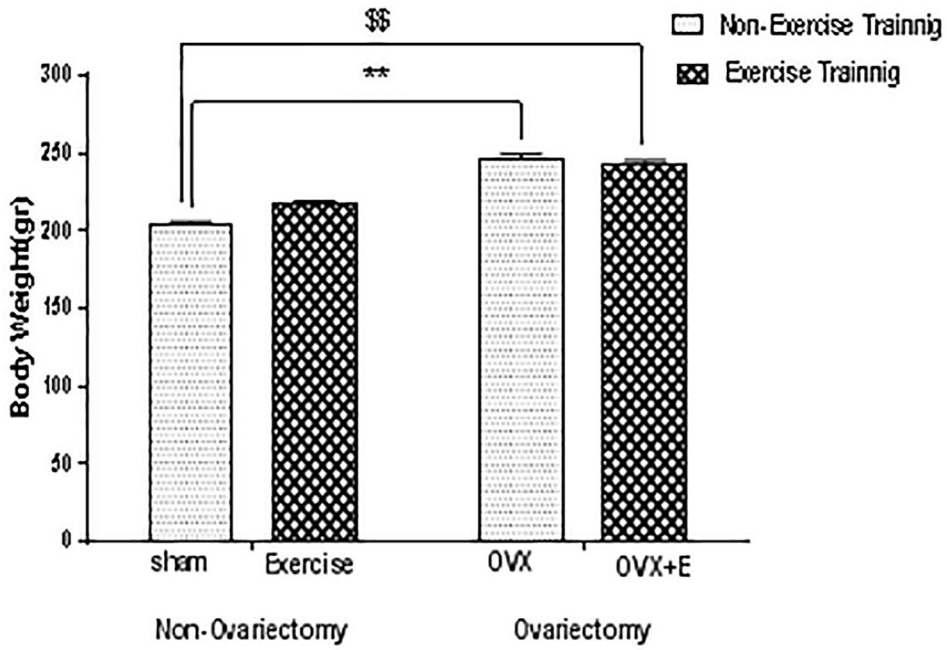

Fig. 1 The mean ± SEM of body weight in the studied groups at end of experimental period. **For $p \leq 0.01$, (OVX vs. Sham-control); $^{\$\$}$for $p \leq 0.01$ (OVX + E vs. Sham-control)

Table 2 The mean ± SEM of lipid profile in experimental groups at the end of study

	Sham-control	Exercise training	OVX	OVX + E
TG(mmol/L)	36.83 ± 3.55	39.83 ± 2.60	82.33 ± 12.38**,$^{\$\$}$	71.33 ± 9.29†
TC(mmol/L)	95.5 ± 1.33	95.83 ± 3.67	130.66 ± 3.82****,$^{\$\$\$\$}$	108 ± 3.53$^{\#\#\#}$
LDL(mmol/L)	57.35 ± 1.62	64.95 ± 3.51	68.035 ± 2.34	68.12 ± 3.94
HDL(mmol/L)	32.33.8 ± 1.56	37.66 ± 2.09	34.66 ± 2.5	34.33 ± 1.34

TG triacylglycerol, *TC* total cholesterol, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein

**, ****For $p \leq 0.01$, $p \leq 0.0001$, respectively (OVX vs. sham-control)

$^{\$\$, \$\$\$\$}$ For $p \leq 0.01$, $p \leq 0.0001$, respectively (OVX vs. exercise training)

†For $p \leq 0.05$ (OVX + E vs. sham-control)

$^{\#\#\#}$For $p \leq 0.001$(OVX + E vs. OVX)

significantly increased in OVX + E group compared to the sham-control group ($p < 0.05$, Table 2). The results showed that TC levels were significantly decreased in OVX + E group in comparison to OVX rats ($p < 0.001$, Table 2). No significant difference was observed among groups in the other lipid profile levels.

The results of liver enzymes (AST and ALT) revealed that the activity of liver enzymes significantly increased in the OVX + E group compared to sham and exercise training groups (Fig. 2). There was no significant difference between groups in liver enzymes levels in comparison with each other.

The hepatic expression of FXR is presented in (Fig. 3a). Data analysis revealed that the relative expression of FXR significantly down-regulated in the liver of OVX rats compared to the exercise training group ($P < 0.05$). In this research results showed that the hepatic expression of FXR was enhanced by exercise training compared to other groups, but this up-regulation was not significant; and the expression of FXR in OVX + E rats was also higher than that in the OVX rats, though this up-regulation was not significant either.

The results showed that ovariectomy decreased the hepatic expression of SHP in OVX group compared to the exercise training group ($P < 0.01$)(Fig. 3b). It was also reported that exercise training for eight weeks could up-regulate the SHP expression in OVX + E group compared to those in the OVX group, though this increase was not significant.

Also, data analysis of CYP7A1 gene expression showed that ovariectomy could decrease the hepatic expression of CYP7A1 gene in OVX rats compared to exercise training group ($P < 0.05$) (Fig. 3c). There was no significant difference between other groups on CYP7A1 gene expression.

Moreover, we examined the simple correlation between FXR, CYP7A1 and SHP gene expression. There was a

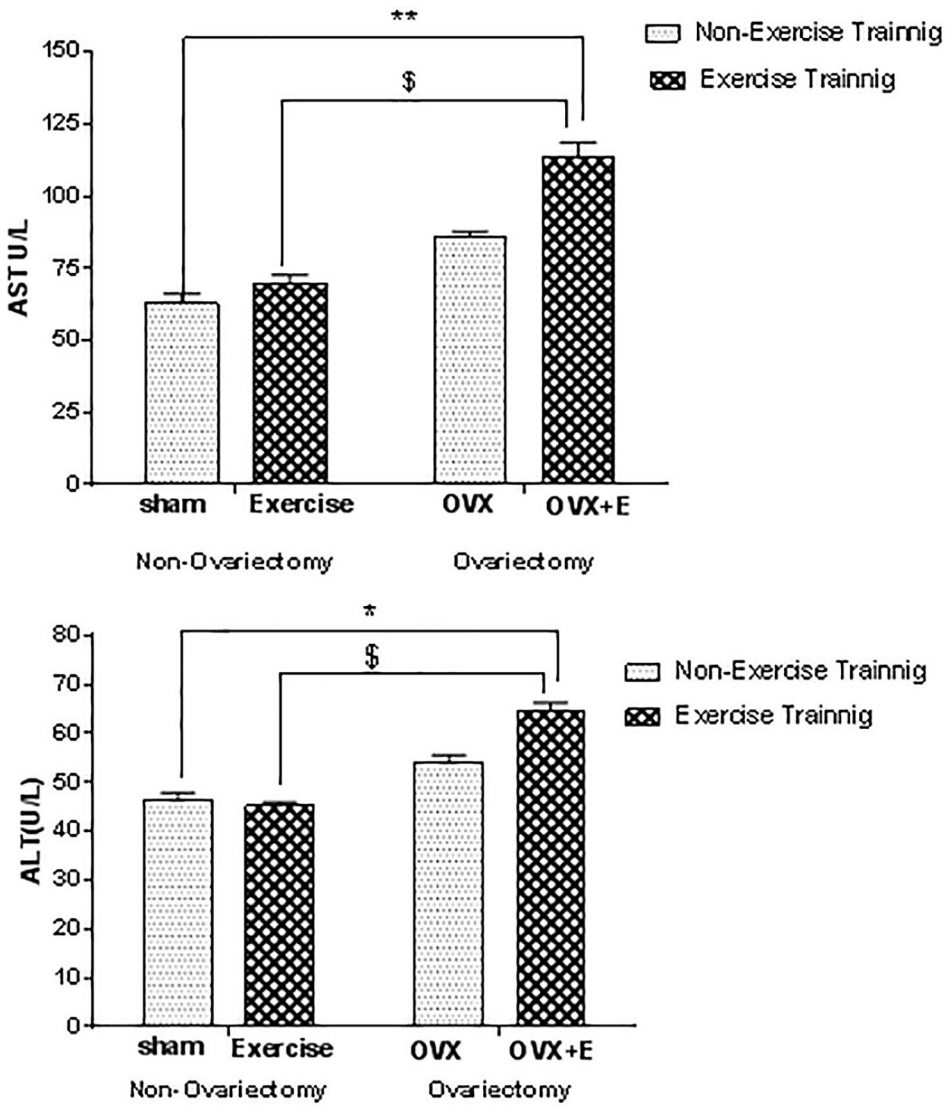

Fig. 2 The mean ± SEM levels of AST and ALT in experimental groups. *, ** for $p \leq 0.05$ and $p \leq 0.01$, respectively (OVX + E vs. Sham-control); $^{\$}$for $p \leq 0.05$ (OVX + E vs. Exercise training)

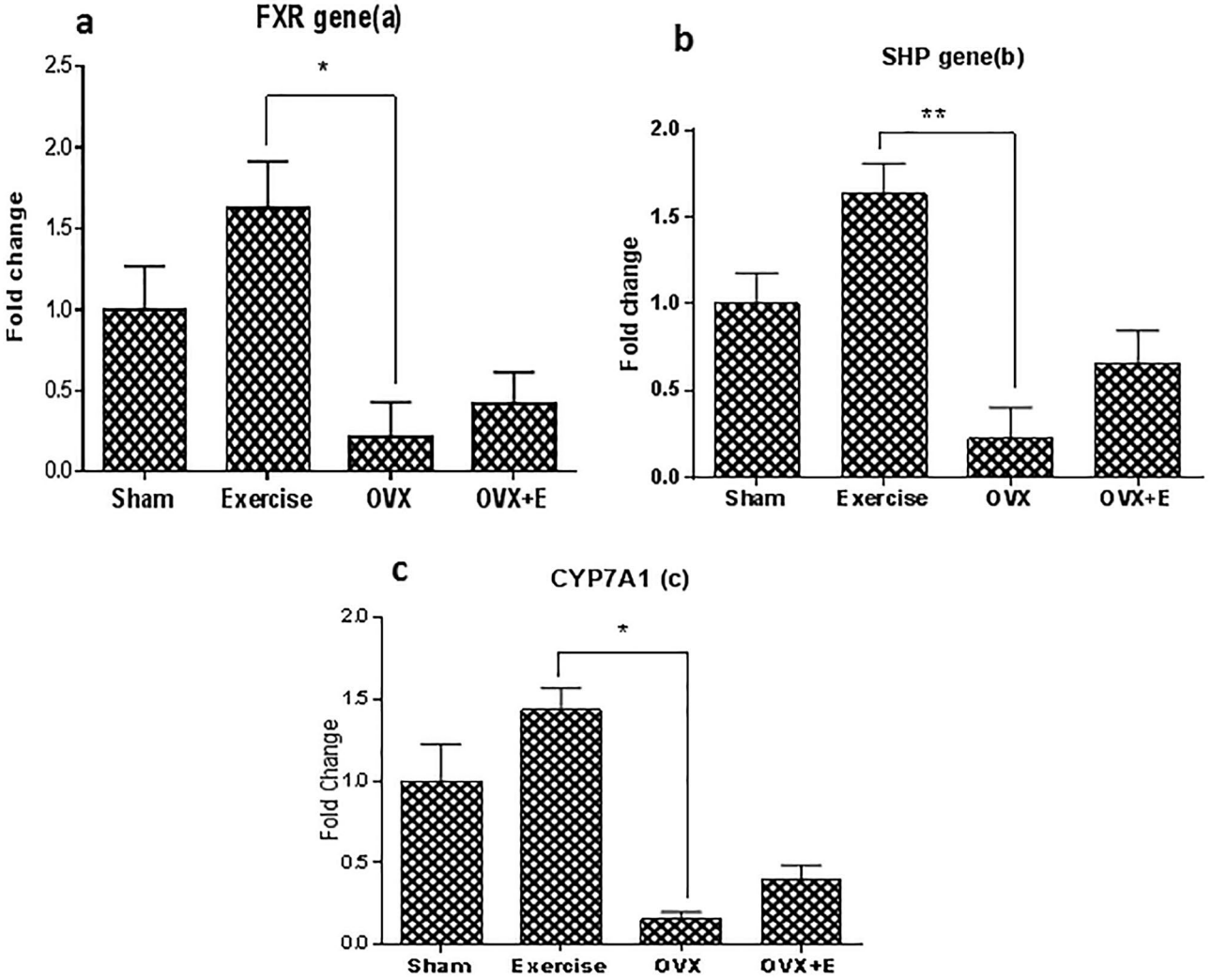

Fig. 3 Expression of FXR (**a**), SHP (**b**) and CYP7A1 (**c**) genes in the liver of the different experimental groups. *, **Significant difference compared with exercise training group ($p < 0.05$) ($p < 0.01$)

significant positive correlation between FXR with SHP levels ($r = 0.56$, $P = 0.0095$).

Discussion

The results of this study show that ovariectomy could increase total body weight. While exercise training could not decrease the body weight gain in ovariectomized rats. These findings were consistent with the results of Pighon et al., Hao L et al. and farahanak [20, 21, 36]. indicating that exercise could not significantly improve body weight gain after ovariectomy. in contrast Like Hao et al. [36] showed that an 8-week moderate exercise program could improve body composition and decrease body weight in ovariectomized rats [36]. Based on previous research, it is assumed that exercise could improve visceral fat in OVX rats and improve body composition and reduce the waist-to-hip ratio in postmenopausal women [12, 37]. Moreover, several reports have suggested that aerobic exercise training could reduce fat deposition, although it could not alter weight gain induced by ovariectomy [24, 36, 37]. In the present study, the body weight has not been changed significantly in OVX rats through exercise, however, it is difficult to find whether weight loss in OVX rats is related to the fat wight or the other components. Also, exercise training has increased the body weight in healthy rats but this weight gain was not significant. It appears that exercise in healthy rats leads to a compensatory increase in muscle weight.

Previous studies showed that estrogen deficiency-induced fat gain and plasma lipid disorder [38], and exercise training could improve lipid profile and cholesterol metabolism [24, 39]. In this ovariectomy, the study increased the plasma levels of TG and TC. However, exercise training reduced

TC in the OVX rats, but could not alter TG, HDL, and LDL plasma level. There are some reports indicating that HDL is enhanced by regular exercise in human [40] and animal subjects [41], but some other studies found that exercise training did not improve HDL, in OVX animals [37, 42] and women in postmenopausal [43]; in line with present reports, studies also showed that TC was reduced and TG was not changed in OVX animals and in postmenopausal women through exercise training [44, 45]. Sock nago et al. [46] found that ovariectomy increases the plasma level of cholesterol by down-regulating the hepatic expression of the LDL receptor, so the elevated TC levels could be due to a dwindle in the uptake of cholesterol from circulation in OVX animals [46]. Ovarian resection causes lipid profile disturbance and this may increase the level of liver enzymes in the plasma [47] and cause liver damage [48]. In the present study, ovariectomy increases ALT and AST enzymes in plasma and exercise increases the level of these enzymes in ovariectomized rats, which is disagreement with the results obtained by Buniam et al. [15] indicating exercise could reduce liver enzymes in plasma. Despite many studies in this field, there is no consensus that exercise can improve the levels of liver enzymes in ovariectomized rats. Some of these studies have shown that exercise can increase [49] or decrease [14] the level of these enzymes. This disagreement in results may be due to a variety of reasons such as different exercise protocols and sampling time [49].

The liver is a major organ in cholesterol metabolism. FXR/SHP/CYP7A1 pathway converts extera cholesterol to bile acid and prevents cholesterol accumulation in liver. The results of this study indicated that hepatic expression of FXR, SHP, and CYP7A1 significantly down-regulated in OVX rats. These findings are in agreement with the previous studies reporting that ovariectomy decreases the gene expression involved in cholesterol metabolism [17, 27, 50]. Wang et al. [27] showed that the expression of SHP is down-regulated in rats by ovariectomy [27] and estrogen deficiency is associated with FXR low expression [19]. It has been reported that CYP7A1 transcription decreases in rats and mice by ovariectomy [16–19], as a result cholesterol elimination via bile acid formation decreases. FXR is believed to be involved in reverse cholesterol transport, and it delivers cholesterol from peripheral tissues to the liver for biliary disposal and consequent fecal elimination [51]. Thereby, reduced FXR expression could induce lesser oxidation and fat removal consequently it causes dyslipidemia, decreased lipolysis, and more body weight gain [30]. Downregulation of CYP7A1 via FXR/SHP signaling after OVX leads to lowerd cholesterol catabolism and excretion from the liver, as a result, plasma cholesterol, and triglyceride increase. In normal conditions, additional regulation of CYP7A1 happens to modulate bile acid exertion and to decrease plasma cholesterol; It also functions as a protection against cholesterol accumulation and liver injury [52–54], So it seems that ovariectomy in the present study disturbs this signaling pathway. Exercise training in the present study could not modulate the expression of FXR,SHP,CYP7A1 genes in OVX rats and after 8 weeks aerobic exercise SHP, FXR, and CYP7A1 increases in OVX rats but not to a significant level. In the liver SHP and CYP7A1 has an inverse relationship but in the current study, this inverse relationship has not been reported. In Farahnak et al. [21] study, exercise could not change FXR gene expression but SHP and CYP7A1 increased and plasma cholesterol improved, they have shown that SHP and exercise have an estrogen effect and reduce cholesterol in the liver in form of bile acid, so higher CYP7A1 expression prevents the liver from cholesterol accumulation through regular exercise training; It is also a compensatory response to eliminate cholesterol from the liver; therefore exercise training is considered to be an effective factor in the reduction of plasma cholesterol. Moreover Similar to the results of this study, it was reported that the gene expression involved in the bile acid metabolism, such as CYP7A1 was not affected by exercise [26, 55] and it seems that This gene needs a stronger stimulus to respond. The present study depicts some positive effects of exercise training on plasma cholesterol concentrations (TC) and no effects of exercise training on gene expression of key markers which are involved in hepatic cholesterol metabolism either in Ovx or Sham animals. Since the Cholesterol transport across the liver is largely altered by the decrease of estrogens, therefore not any changes in genes involved in RCT process proved not to be the result of a beneficial effect of exercise training on FXR/SHP/CYP7A1 pathway. It is interesting that the plasma cholesterol concentrations reduced in Ovx rats after 8 weeks of exercise training while the gene expression did not change. It is possible that this pathway needs a longer duration of time or intensity to respond to a training stimulus. In addition, it was shown that non-hepatobiliary is also a mechanism responsible to reduce cholesterol from peripheral tissue and plasma; so it is possible that exercise training reduces plasma cholesterol levels through different mechanisms or any changes occurred at post-transcriptional levels are due to several post-transcriptional mechanisms which have been implicated in the regulation of CYP7A1 /SHP/FXR expression. Moreover, lowered cholesterol might have happened by another pathway in the liver like LXR/CYP7A1 pathway that was not evaluated in this study [45].

Taken together, the present results suggest a positive association between body weight change, TG,HDL,LDL plasma, and gene expression of CYP7A1,SHP,FXR receptors after ovariectomy, and FXR/SHP/CYPA71 pathway could not be upregulated by exercise after ovariectomy. According to the results, exercise protocol could not be effective in body weight, lipid profile except for TC in the ovariectomized

rats which could be due to the dominant effect of ovariectomy and impairment of FXR/SHP/CYP7A1 pathway to reduce cholesterol. So exercise could not compensate for the impairment which happened after estrogen deficiency and cholesterol excretion in the liver, due to ovariectomy by FXR/SHP/CYP7A1 pathway. It appears that exercise can improve cholesterol metabolism by the genes that are involved in RCT process when they significantly change body weight and lipid profile.

Conclusion

Based on the results of this study, it can be concluded that ovariectomy down-regulated the expression of genes involved in the cholesterol metabolism and subsequently increased plasma TC and TG levels. Although in this study the exercise protocol could improve TC levels in ovariectomized rats, it did not alter the hepatic expression of genes involved in the cholesterol metabolism in these animals. These effects may be due to other regulatory mechanisms that have not been examined in this study. It is also suggested that exercise training with different intensity and duration or incorporating diet may be effective to reduce the undesirable impacts of ovariectomy by this pathway in the liver. So other kinds of gene expression and different intensity of exercise and mechanism responsible for cholesterol metabolism are recommended to be measured in further experiments and studies.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval All steps of the study were carried out in accordance with "Guiding Principles for the Care and Use of Research Animals" approved by the Ethical Committee of University of Mazandaran.

Informed consent None.

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