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The Effect of Eight Weeks of Combined High-Intensity Interval Training on Intrahepatic PGC-1α and PEPCK Protein in Male Rats with Non-Alcoholic Steatohepatitis

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ABSTRACT

Introduction: Non-alcoholic fatty liver disease (NAFLD) is a globally prevalent, non-communicable metabolic disease. Therefore, treatment strategies for this disease are essential. This research aimed to examine the effect of eight weeks of combined high-intensity interval training on intrahepatic PGC-1α and PEPCK protein in male rats with non-alcoholic steatohepatitis.

Methods: A total of 40 rats aged 6-8 weeks were divided into two groups: healthy (n=20) and high-fat diet (HFD) (n=20). The HFD group was randomly divided into control-patient (n=9) and training-patient (n=9) after eight weeks and assurance of disease induction. The healthy group was divided into control-healthy (n=9) and training-healthy (n=9). The training group rats performed HIIT in aquatic and land environments (Saturdays and Wednesdays in aquatic environments and Mondays on a treadmill). One-way ANOVA and Bonferroni's *post hoc* test (P<0.05) were performed to determine the difference between groups.

Results: Intrahepatic PGC-1α protein levels significantly increased in the healthy control group compared to the patient control (P=0.04). In addition, intrahepatic PEPCK protein levels significantly increased in the control-healthy group (P=0.01), training-healthy (P=0.002), training-patient group (P=0.03), and training-patient (P=0.03) group compared to the control-patient group.

Conclusion: Based on the results, combined high-intensity interval training can effectively regulate the PGC-1α/PEPCK mechanism, a potential mechanism for regulating glucose and lipid metabolism in the liver. However, further research is needed in this regard to obtain conclusive results.

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Introduction

Peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α) is regarded as one of the biochemical factors critical in the development of nonalcoholic fatty liver disease (NAFLD), a disease spectrum ranging from nonalcoholic fatty liver without inflammation to nonalcoholic steatohepatitis (NASH). Alternatively, NASH can lead to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). There are substantial improvements in the clinical management of these diseases; however, a partial understanding of the molecular mechanisms underlying the onset of these conditions slowed the development of effective therapeutic strategies. In this vein, molecular modifications leading to altered gene expression and subsequently contributing to disease development could be potential targets to improve hepatic functions (1, 2). PGC-1α, as a transcriptional activator, regulates hepatic energy metabolism, including hepatic mitochondrial respiration, fatty acid oxidation, and gluconeogenesis (2).

Apart from fatty acid oxidation, PGC-1α can also upregulate hepatic phosphoenolpyruvate

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carboxykinase (PEPCK), which is involved in developing hepatic steatosis. In a fasting state, rats with hepatic PEPCK block developed severe hepatic steatosis due to inhibiting the Krebs cycle and oxidation of fatty acids. Moreover, rats lacking hepatic PEPCK were significantly defective in glucose utilization in the liver and skeletal muscles, and it is thought that impaired PGC-1α/PEPCK signaling in the liver plays a key role in developing hepatic steatosis (3).

Given the effective mechanisms mentioned in non-alcoholic steatohepatitis, researchers disagree on the best training protocol regarding type, intensity, volume, and rest period (4). Considering the advantages of high-intensity interval training, this type of training in the land environment may cause limitations in some statistical populations, including obese patients and patients with non-alcoholic steatohepatitis (5).

Training in the aquatic environment due to the unique properties of water, such as inertia or buoyancy and weightlessness, has been introduced in the research as a suitable option for these clinical communities. However, contradictory results have been reported on improving the symptoms of the disease (5). On the other hand, the training in water, like on land, reduced inflammatory indicators and increased mitochondrial efficiency and volume (6).

Despite these results from the previous research, researchers disagree on the best training protocol. Therefore, the literature review is on the benefits of aquatic training and the public's particular interest in performing aquatic and land training simultaneously, and there is no research on the combined effect of high-intensity interval training in the aquatic and land environments on patients with NASH. Thus, this study explored the impact of eight weeks of combined high-intensity interval training on intrahepatic PGC-1α and PEPCK protein in male rats with non-alcoholic steatohepatitis.

Materials & Methods

The developmental-fundamental study was semi-experimental. This research selected 40 male Sprague-Dawley rats (age: 8 weeks) with an average weight of 230±20g. The rats were maintained in the animal house of Shiraz University with an ambient temperature (22-24°C), humidity of 45%, and a light-dark cycle of 12-12h.

Animals' access to standard and free food was made by Shiraz University of Medical Sciences. In addition, water was provided freely in 500ml bottles for laboratory animals. The ethical principles were considered in compliance with the principles of working with laboratory animals based on the Helsinki Declaration and approved by Shiraz University of Medical Sciences with the code of ethics IR.SUMSAEC.1402.016.

The selected rats were then divided into two groups of 20 animals. The first group, the healthy group, received standard food (n=20). The second group was exposed to a high-fat diet (HFD) for eight weeks to induce NASH (n=20).

The high-fat diet was provided as an emulsion for eight weeks) (n=20). The high-fat emulsion comprised 77% fat, 14% protein, and 9% carbohydrates. The ingredients of this emulsion included corn oil, sucrose, cholesterol, milk powder, sodium deoxycholate, propylene glycol, Tween 80, a mixture of vitamins and minerals, and distilled water. In this emulsion, proteins were supplied by milk powder, carbohydrates by sucrose, and fats by corn oil and cholesterol powder (7).

Rats in the healthy group (n=20) were also given equal salt solution (saline) daily. The patient group (high-fat diet) was randomly divided into two groups after proving the induction of the disease in rats (the relevant findings are given in the results section): control-patient (n=9), training-patient (n=9), and the healthy group was randomly divided into two groups: control-healthy (n=9) and training-healthy (n=9).

The patient groups' high-fat diet continued until the training protocol's end. Rats in the training groups had two weeks to learn about the animal pool (160cm diameter, 80cm height) and the rodent treadmill after group categorization and before the leading training started (8).

HIIT Swimming Workout

This training was performed twice weekly (Saturdays and Wednesdays) for eight weeks. In Week One, rats were carefully and gently placed in the animal pool with a depth of 50cm and an average temperature of 30 ± 0.5°C, and they swam intermittently at an arbitrary speed for 20 minutes. As the rats became familiar with the animal pool, they were taken out of the water several times after swimming for one minute by the rest plate and put back in the water in the second week.

About 48 hours after the last familiarization session, rats in the training group first warmed up for five minutes and then performed the main HIIT swimming workout, including 20 intervals of 30 seconds of swimming with 30 seconds of rest between each interval. After five minutes of cooling, all the rats were completely dried and transferred to their cages upon completing each training protocol.

In the interval training, the load in Week One comprised a weight equal to seven percent of each rat's body weight, and 1% was added every week. Thus, in Week Eight, rats swam with a weight equal to 14%, tied to the end of their tail (Table 1) (9). The training was performed in the evening (the best time for training for the natural activity rhythm) (10).

Table 1. HIIT swimming workout protocol (every other day, even days)

Week	Number (interval)	Attempt duration (seconds)	Rest period (seconds)	Amount of overload (percentage of body weight)
Week One	20	30	30	7
Week Two	20	30	30	8
Week Three	20	30	30	9
Week Four	20	30	30	10
Week Five	20	30	30	11
Week Six	20	30	30	12
Week Seven	20	30	30	13
Week Eight	20	30	30	14

HIIT on the Treadmill

The training group performed a high-intensity interval protocol once a week (on Mondays) over eight weeks of the experimental period. Two weeks before the start of the study, the animals were acclimatized by running on the treadmill for 20 minutes at a speed of 10m/min through a low-intensity running protocol every day.

According to Table 2, in the first session, the training intensity was 16m/min for 3 minutes, followed by active recovery of 10 m/min for two minutes. The activities were performed in four sets. With the passage of time, the training intensity was increased so that from Week 5 to 8, the training intensity was 37m/min for three minutes, followed by active recovery with an intensity of 28m/min for two minutes (11, 12).

Table 2. Land training protocol

Week	Duration of main training (min/day)	Vo_{2max}(Percentage of intensity)	No. of sessions per week	Incline
1	20	10 m/min (2 min. recovery) 16 m/min (3 min. training)	1	0°, 60%Vo_{2max}
2	20	16 m/min (2 min. recovery) 23 m/min. (3 min. training)	1	0°, 70%Vo_{2max}
3	20	22 m/min (2 min. recovery) 30 m/min (3 min. training)	1	0°, 80%Vo_{2max}
4	20	22 m/min (2 min. recovery) 30 m/min (3 min. training)	1	0°, 80%Vo_{2max}
5-8	20	28 m/min (2 min. recovery) 37 m/min (3 min. training)	1	0°, 90%Vo_{2max}

The rats' weight was initially measured from the first research day and every Friday evening, and this process continued until the end of the research and the dissection day. The average weight of each group was calculated and recorded after obtaining the rats' weight in each group.

The findings related to the average weight of the research groups are reported in Figures 1 and 2. The healthy-control and patient-control groups had no training protocol during the intervention period.

The rats were injected intraperitoneally with a combination of ketamine (30-50 mg/kg of body weight) and xylazine (3-5 mg/kg of body weight) and anesthetized 48 hours after the last training session to eliminate the acute effects of training and the uncontrolled stressing variables in animals during the implementation of the training protocol. Then, the liver tissue was extracted from the animals, washed in physiological serum, and immediately frozen at -80˚C.

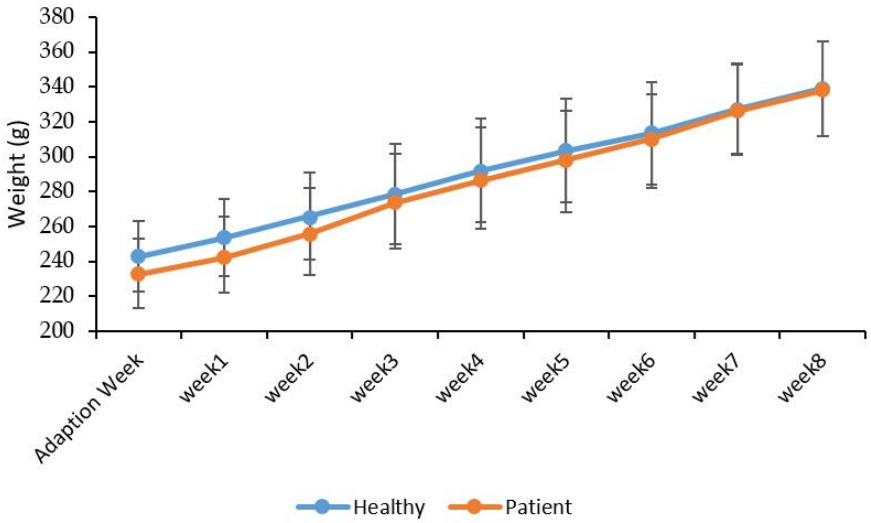

Figure 1. Mean ± standard deviation of the weight changes of the healthy (n = 20) and high-fat diet (HFD) groups in the first eight weeks of fatty liver induction (at the beginning, one week was considered for adaptation of the rats to the laboratory environment and diet).

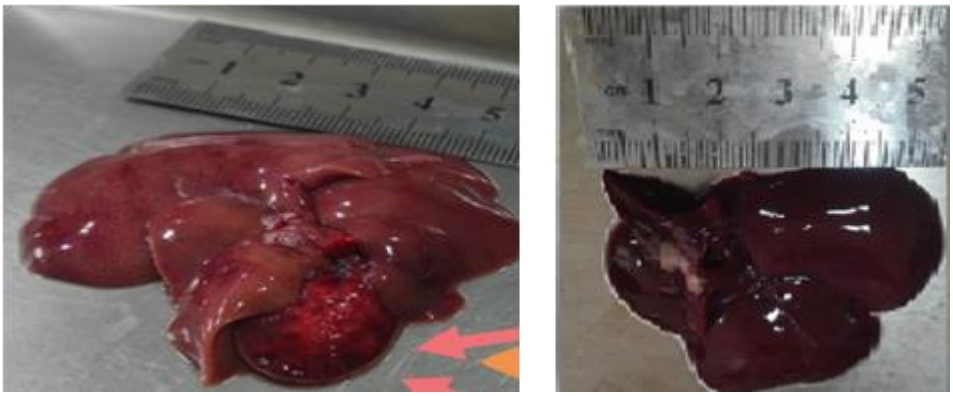

Figure 2. Comparison of the appearance of healthy liver (A) and fatty liver (B), immediately after killing in the diseased and healthy group

Biochemical and Analysis Methods

The liver tissue was separated to measure the tissue protein, washed in normal saline solution, and immediately stored in a nitrogen tank at a temperature of -196˚C. Then, the prepared liver tissue was transferred to the laboratory to perform the western blot technique and the final analyses of PEPCK and PGC-1α proteins. In general, the steps of the western blot technique included tissue lysing, determination of protein concentration by Bradford, preparation of different concentrations of BSA to draw a standard curve, protein concentrations, water and sample buffer, sample preparation, electrofusion on SDS PAGE gel, preparation of solutions, test method and making of lower and upper gel, electrophoresis on SDS page gel, western blot or immunoblotting, transfer step from gel to paper, blocking step, incubation step with primary antibody (β-Actin (C4): sc-47778, PEPCK (F-3): sc-271029) from Santa Cruz Biotechnology, Inc. and (β-Actin (C4): sc-47778, Anti-PGC1 alpha antibody: Q86YN6) from abcam, incubation stage Using the secondary antibody (m-IgGκ BP-HRP: sc-516102, mouse αanti-rαββit IgG-HRP: sc-2357), the detection stage, the stage of film emergence in the dark room, and the Striping method, respectively.

ALT and AST Measurement Method

First, blood samples were taken from the hearts of rats in the amount of 5cc. Each sample was immediately transferred inside the activator chelate gel test tube and then centrifuged at a speed of 3000rpm and a temperature of 18°C for 20 minutes. Then, blood serum was transferred to 2cc microtubes by sampler and kept at minus 20°C for final analysis. AST and ALT enzymes were measured from blood serum by diagnostic kits from Pars-Azmon using the photometric laboratory method (order number: 118400).

The descriptive statistics used measures of dispersion, mean, and standard deviation. In the inferential statistics, the Shapiro-Wilk test was used to determine the normal distribution of the collected data. A one-way analysis of variance was used in the normal distribution of the data. When the differences were significant, Bonferroni's *post hoc* test was used to determine the exact location of the differences. The level of significance was considered <0.05. All statistical procedures were run using SPSS version 26 software, and Excel2019 software was used to draw figures.

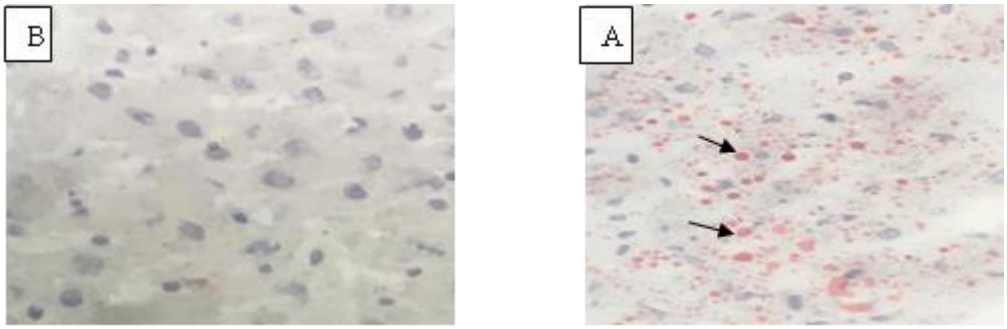

Figure 3. Staining of liver tissue by Oil red O; A: Fatty liver, lipids are red. B: Healthy liver - Not taking on any red color due to the absence of fat.

Results

Figure 1 shows the mean±standard deviation of the weight changes of the healthy (n=20) and high-fat diet (HFD) (n=20) groups during eight weeks of high-fat diet induction. The first week was considered for the adaptation of the rats to the laboratory environment, followed by eight weeks of high-fat diet induction.

Four rats were randomly sacrificed from the patient (n=2) and healthy (n=2) groups at the end of the induction of non-alcoholic steatohepatitis. As shown in Figure 2, the diseased liver appears white owing to a high accumulation of triglycerides, whereas the healthy liver appears red as there is no accumulation of triglycerides.

A pathologist inspected the pathological and biochemical properties of the liver tissue after examining the appearance of the liver. Figure 3 presents the results of Oil red O staining of the diseased and healthy liver tissues. The number of red dots in the diseased liver sample indicates high triglyceride accumulation in this tissue. However, no red dots were observed in the healthy liver sample, indicating the absence of triglyceride accumulation in the healthy liver tissue.

Table 3. Results of tissue and blood tests to show non-alcoholic steatohepatitis (NASH) by a pathologist who was completely unaware of the research method and purpose.

Rat No.	Steatosis grade	Inflammation grade	Fibrosis stage	ALT	AST
1 (Patient)*	2.33	0.3	2	64.54	60.9
2 (Patient)*	2.75	0.9	2	69.7	67.84
1 (Healthy)*	0	0	0	46.3	41.21
2 (Healthy)*	0	0	0	40.39	38.41

As shown in Table 3, in the diseased samples of 1 and 2 after eight weeks of HFD, steatosis and inflammation grades and serum ALT and AST values increase. In addition, liver histological analysis showed that the rats' liver was in the second fibrosis stage. Figure 1 shows the weight of the rats in the research groups after proving NASH.

Considering the average weight of each group in the whole period of training intervention, no

significant difference was found between the training-patient and training-healthy groups (P=0.038) as well as the control-patient (P=0.027) and control-healthy groups (P=0.031 in Week Seven). Thus, the average weight of the training-patient group went through a significant decrease compared to the control-patient group (P=0.027).

Table 4. Descriptive statistics of the research variables

Variable	Group	Mean	SD	n
PGC-1α (pg/ml)	Control-Healthy	1	0	2
	Training-Healthy	0.975	0.015	2
	Control-Patient	0.525	0.125	2
	Training-Patient	0.67	0.04	2
PEPCK (pg/ml)	Control-Healthy	1	0	2
	Training-Healthy	1.39	0.042	2
	Control-Patient	0.325	0.19	2
	Training-Patient	0.865	0.007	2

In Table 4, descriptive data are displayed, including the mean, standard deviation, minimum, maximum, and the number of animals for each dependent variable in experimental groups.

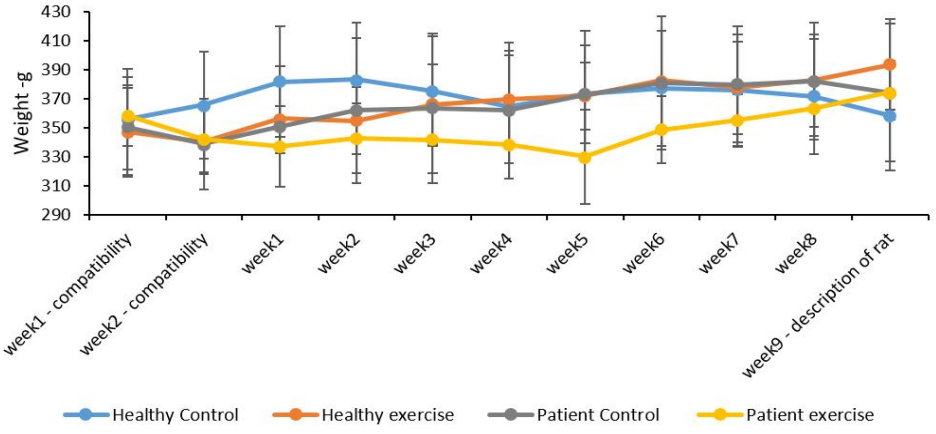

Figure 4. Mean ± standard deviation of the weight changes of the four experimental groups in the second eight weeks following the emergence of non-alcoholic steatohepatitis (NASH) (Initially, two weeks were considered for adaptation of the rats to the training environment and then the main training was performed from weeks one to eight according to the protocol. The ninth week was the week for rats' dissection.

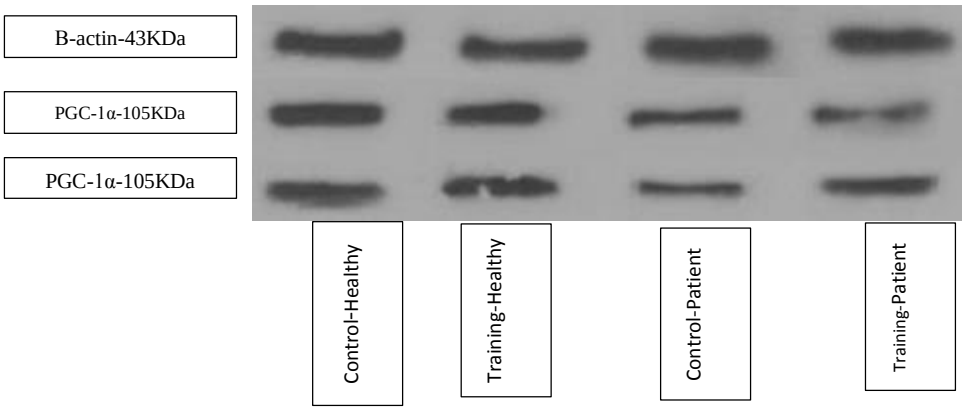

Figure 5. Results of the Western blot for intrahepatic PGC-1α protein; the concentration of this protein is determined according to its bandwidth and color intensity compared to beta-actin in the liver.

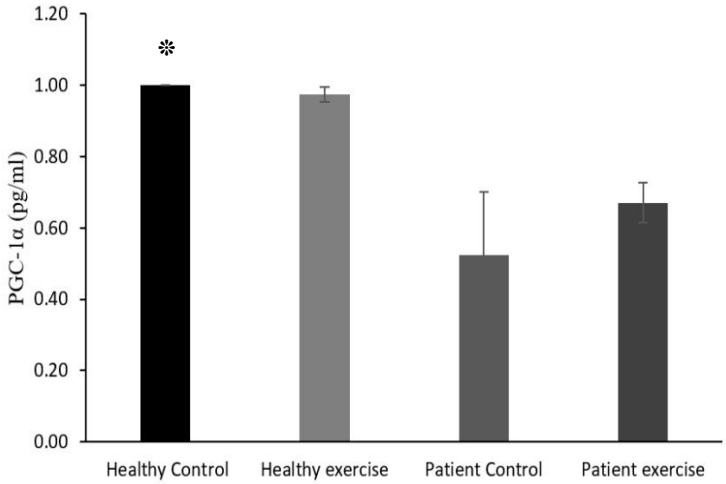

Figure 6. Intrahepatic PGC-1α protein levels
*: significant increase in the healthy control group compared to the patient control (P=0.042)

As shown in Figure 6, based on the results of a one-way analysis of variance, there is a significant difference between the mean of PGC-1α (P=0.017, η^2=0.075). Moreover, based on Bonferroni's post hoc test results, these differences in the given variable are significant between the control-healthy and control-patient groups (P=0.042, η^2=0.061). Besides, the training-patient group had a non-significant increase of 21.641% compared to the control-patient group (P=0.9, η^2=0.002) by examining the mean scores.

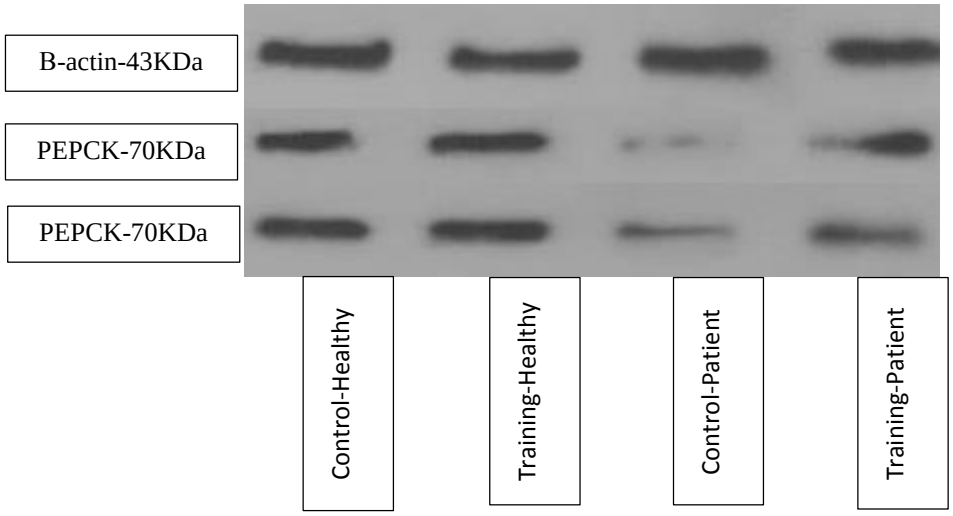

Figure 7. Results of the Western blot for intrahepatic PEPCK protein; the concentration of this protein is determined according to its bandwidth and color intensity compared to beta-actin in the liver.

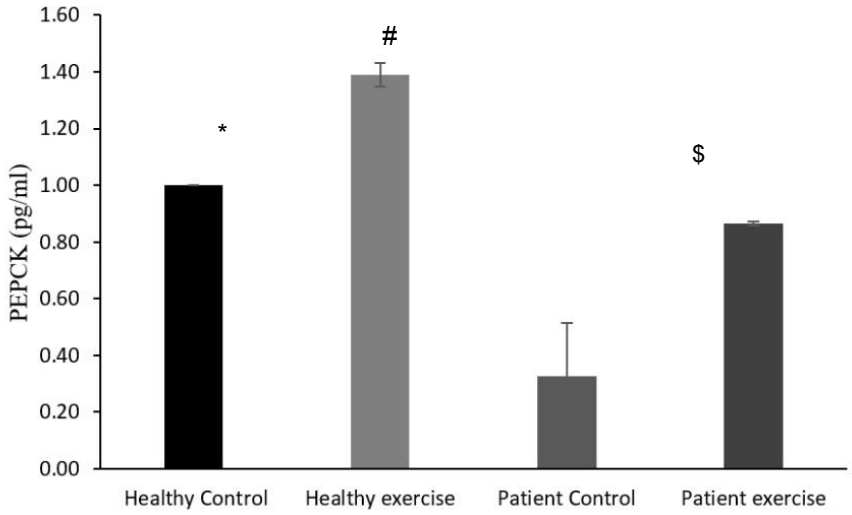

Figure 8. intrahepatic PEPCK protein levels
*: significant increase in the healthy control group compared to the patient control (P=0.014);
#: significant increase in the healthy training group compared to the control-patient (P=0.002) and Training-Patient (P=0.035) groups;
$: significant increase in the Training-Patient group compared to the Control-Patient (P=0.032)

Figure 8 shows a significant difference between the PEPCK mean based on the results of one-way ANOVA (P=0.002, η^2=0.081). According to Bonferroni's *post hoc* test, this difference is related to a significant increase in the control-healthy group compared to the control-patient (P=0.014, η^2=0.074), a significant increase in the training-healthy group compared to the control-patient (P=0.002, η^2=0.085) and training-patient (P=0.035, η^2=0.068) groups, and a significant increase in the training-patient group compared to the control-patient group (P=0.032, η^2=0.065).

Discussion

This study aimed to examine the effect of eight weeks of combined HIIT on intrahepatic PGC-1α and PEPCK protein in male rats with non-alcoholic steatohepatitis. Regarding the values of PGC-1α, the current study's findings showed that this protein increased significantly in the healthy control group compared to the patient control group. Besides, a non-significant increase in this protein was observed in the training-healthy and training-patient groups compared to the control-patient group.

In line with the results of the current study, Norheim et al. (2014) evaluated the effect of 12 weeks of endurance training on a pre-diabetic human model and found that PGC-1α in visceral adipose tissue had a non-significant increase. This small amount significantly affected the regulation of the biochemical metabolism of adipose tissue (13).

Vainshtein et al. (2015) were also in line with the current research's findings, so an increase in PGC-1α was evident after sports activity. Eventually, they concluded that sports activity increases PGC-1α and regulates lipid metabolism and blood sugar (14).

Regarding PEPCK in the current study, a significant increase was found in PEPCK in both training-patient and training-healthy groups compared to the control-patient group. In line with the results of the present research, Yeylaghi and Ashrafi et al. (2021) explored changes in PEPCK gene expression in the liver tissue and HOMA-IR following HIIT and consumption of royal jelly consumption in type 2 diabetic rats. Finally, they concluded that HIIT and training+royal jelly consumption can increase the expression of the hepatic PEPCK gene and reduce HOMA-IR (15). Further, Haase et al. (2011) investigated the effect of exercise and fasting-induced adaptations on the PGC-1α/PEPCK pathway and concluded that both chronic exercise and fasting-induced adaptations, as well as a single bout of exercise can increase the activity of PGC-1α/PEPCK pathway. Ultimately, they concluded that the PGC-1α protein is a key factor in regulating liver metabolism (16).

Fletcher et al. (2014) examined the effects of different training methods on the level of mitochondrial respiration and liver metabolism. The expression of PGC-1α and PEPCK genes in the liver increased regardless of the training method compared to the control group. Regardless of its type, exercise could increase mitochondrial respiration and improve liver metabolism (17).

Kazemi Nesab et al.'s (2020) results contradict this study's. They aimed to explore the effect of eight weeks of aerobic training on the expression of LXRa, PEPCK, and G6PC2 mRNA genes in the liver tissue of pre-diabetic obese rats. The liver LXRa and ABCA1 amounts in the trained rats were significantly higher than in the control group.

The relative expression of critical genes in gluconeogenesis, including G6PC2 and PEPCK, in the livers of the trained rats was significantly decreased compared to the control group. Moreover, aerobic exercise reduced steatosis and liver enzymes in pre-diabetic rats (18).

Kazemi Nasab's research and this research's results are inconsistent because of the type of training used in both studies. In Kazemi Nasab's study, moderate-intensity endurance training was performed five days a week, one hour of training per session for eight weeks, while the training protocol of the current research comprised three days of HIIT per week, and each session continued for 20 minutes.

Compared to moderate-intensity training, high-intensity interval training (HIIT) leads to the further discharge of stored carbohydrates in the liver, and as a result, enzymes effective in gluconeogenesis, including PEPCK, are activated and produced more during the post-exercise period. In addition, the difference in the disease model induced in these studies can be mentioned among the other reasons for the contradiction in the findings of the two studies, so that the model of the disease induced was pre-diabetes in Kazemi Nasab's study. In contrast, in the present study, it was non-alcoholic steatohepatitis induced by high-fat diet consumption.

Shokrollahi et al. (2019) suggested that the increase in hepatic glucose release due to dysfunction of enzymes effective in the hepatic gluconeogenesis process is one of the significant characteristics of type 2 diabetes. They conducted a study attempting to examine the effect of 12 weeks of resistance training on the expression of glucose six phosphatase (G6Pase) and PEPCK in the liver cells, as well as glucose levels and beta cell function in type 2 diabetic rats.

This study revealed a significant decrease and a non-significant decrease in the expression of glucose six phosphatase and PEPCK genes in the trained rats compared to the control rats, respectively. The type of training protocol can be mentioned among the reasons for the discrepancy between the recent and current research findings.

In the current study, a high-intensity interval training protocol was used in combined aquatic and land environments, three sessions per week, and each session lasted 20 minutes for eight weeks. However, in Shokrollahi et al., five resistance training sessions per week were used for 12 weeks. Further, in Shokrollahi et al., the disease model was type 2 diabetes with nicotinamide-streptozotocin injection. At the same time, in the current research, it was non-alcoholic steatohepatitis (NASH) induced by high-fat diet consumption. Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme in gluconeogenesis.

PEPCK gene expression is controlled by insulin and glucagon. PGC-1α can rapidly increase the transcription of several transcription factors, such as hepatocyte nuclear factor 4 alpha (HNF-4α) and FOXO, thereby controlling the transcription of gluconeogenesis enzymes such as Phosphoenolpyruvate carboxykinase (PEPCK).

Nonetheless, pancreatic beta cells produce and secrete insulin following a meal, which binds to its receptor and causes AKT phosphorylation, which, in turn, phosphorylates PGC-1α and inhibits its activity, stimulates glycogen synthesis, and inhibits gluconeogenesis (19).

In this study, the protein levels of PGC-1α and PEPCK were significantly reduced compared to other groups due to the high-fat diet consumption of the control-patient group. A potential mechanism can be inhibiting two proteins mentioned above factors by high insulin secretion. On the other hand, performing combined interval training in both aquatic and land environments caused the stimulation of PGC-1α protein, followed by increased activity of PEPCK in the training-patient group.

A significant increase in PEPCK is likely due to energy discharge caused by HIIT in the training-

patient group as compared to the control group, increasing intrahepatic fatty acid oxidation and insulin sensitivity, which leads to improved symptoms of non-alcoholic steatohepatitis, including insulin resistance in these patients (20).

Conclusion

Based on the results, combined high-intensity interval training (aquatic+land) can effectively regulate the PGC-1α/PEPCK mechanism, a potential mechanism for regulating glucose and lipid metabolism in the liver. Nonetheless, further research is needed to obtain conclusive results. Regarding the limitations of the research, there was a lack of control of rats' stress during gavage, training, and anesthesia. The study duration can also be considered as one of the research limitations because no significant difference was found between the weight variables of the research groups. In future studies, caloric restriction can be applied in addition to the training intervention to find out to what extent exercise and caloric restriction play a role in improving the factors related to non-alcoholic steatohepatitis (NASH).

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Conflict of Interest

The authors of this article declare no conflict of interest.

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