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Original article

The effect of running and starvation intervention on atherogenic index and xbp1 gene change in liver endoplasmic reticulum of non-alcoholic fatty liver rats.

Running title: Regulation of endoplasmic reticulum stress-induced apoptotic pathway activity by running and starvation intervention

Mahshad Paziraee

Department of Physical Education and sports sciences, Aliabad katoul Branch, Islamic Azad University, Aliabad Katoul, Iran. mahshad.pazirayi@gmail.com; ORCID: 0009-0003-2993-7286

***Habib Asgharpour**

Department of Physical Education and sports sciences, Aliabad katoul Branch, Islamic Azad University, Aliabad Katoul, Iran. Habib.asgharpour@gmail.com; ORCID: 0000-0002-4116-981x

***Asra Askari**

Department of Physical Education and sports sciences, Gorgan Branch, Islamic Azad University, Gorgan,Iran, askari.asra@gmail.com; ORCID: 0000-0002-4948-9658

Reza Rezaei Shirazi

Department of Physical Education and sports sciences, Aliabad katoul Branch, Islamic Azad University, Aliabad Katoul, Iran, rezaii725@yahoo.com; ORCID: 0000-0002-0657-2865

Neda Aghaei Bahman Beglo

Department of Physical Education and sports sciences, Aliabad katoul Branch, Islamic Azad University, Aliabad Katoul, Iran, nedaaghaei@gmail.com; ORCID: 0000-0003-0972-1288

Corresponding authors:

1.Dr Habib Asgharpour

Address: Department of Physical Education and sports sciences, Aliabad katoul Branch, Islamic Azad University, Aliabad Katoul, Iran

Email: Habib.Asgharpour@gmail.com

2.Dr Asra Askari

Address: Department of Physical Education and sports sciences, Gorgan Branch, Islamic Azad University, Gorgan,Iran

Email:askari.asra@gmail.com

Tel: +989111566028

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Abstract

Background :Running and starvation can have a positive effect on the reticulophagy of the patient's liver tissue. The purpose of this research is the effect of running and starvation intervention on atherogenic index and xbp1 gene change in liver endoplasmic reticulum of non-alcoholic fatty liver rats (NAFLD).

Methods: 30 obese male Wistar rats aged 18-20 weeks with an average body weight of 348 ± 25.53 after one week familiarization with the laboratory environment were randomly divided into 6 groups of 5: 1) starvation, 2 and 3) (3 and 5 days of training), 4 and 5) (3 and 5 days of training with starvation) and 6) the control group were divided. All fatty model animals had free access to water and standard pellets (10 gr of food per 100 g of mouse body weight). The statistical test of one-way analysis of variance was used at a significance level of less than 0.05 and the LSD post hoc test was used among the research groups.

Results: According to the experimental results and statistical analysis of one-way analysis of variance it showed a significant decrease in the ratio of lipoproteins (VLDL/HDL, LDL/HDL) in all experimental groups compared to the control group, and also a significant decrease in the expression of XBP1 and CHOP genes was observed in the groups of 3 and 5 days of exercise alone and with starvation.

Conclusion: Regular exercise for 3 and 5 days per week with starvation can possibly help by reducing the possible activity of genes involved in increasing endoplasmic reticulum stress in NAFLD patients.

Keywords::CHOP; Exercise; Endoplasmic Reticulum; Starvation; Xbp1

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Introduction:

The liver is a complex organ that performs many physiological functions (1), including the synthesis, oxidation, and transport of free fatty acids (FFA), triglycerides (TG), cholesterol, and bile acids (BA). It plays a key role in lipid homeostasis (2). These processes act through pathways that lead to oxidative stress, chronic inflammation, and insulin resistance (3).

The reported prevalence of non-alcoholic fatty liver (NAFLD) in Western countries is between 30 and 46%. This disease has also spread in eastern countries and has become one of the public health concerns in these regions (4). NAFLD includes a spectrum of liver damage from steatosis to nonalcoholic steatohepatitis (NASH), which can lead to fibrosis (5). People with NAFLD are also at increased risk of cardiovascular disease, type 2 diabetes, and obesity-related mortality. The exact mechanisms of NAFLD are still not well understood (6). The "multiple hit hypothesis" is currently the most recognized theory to explain the development and progression of the disease. The initial shock leads to simple steatosis, while the subsequent shocks include mitochondrial dysfunction, oxidative stress, adipocytokine changes, lipid peroxidation, Kupffer cell activation, etc., leading to liver cell inflammation and apoptosis, which in Finally, it leads to simple steatosis (7) .Recently, and based on accumulated data, it has been shown that disruption of endoplasmic reticulum (ER) homeostasis, or ER stress, is involved in both the development of Steatosis and the progression to NASH (8). ER is a membrane-bound organelle that provides a specialized environment for the production and post-translational modification of secretory and membrane proteins, lipid biosynthesis, and intracellular Ca2+ homeostasis (9). Some physiological and pathological conditions, including temperature and pH changes, accumulation Damaged DNA can cause ER stress (10). ER stress can be divided into three types, including the unfolded protein response (UPR), ER overload response, and sterol regulatory elements along with regulatory responses with protein mediator; ER stress is commonly referred to as the UPR and occurs when folded or unfolded proteins in the ER increase and activate a stress signal that is transmitted through the ER membrane to the nucleus (11). Findings show that membrane receptors ERs recognize the onset of ER stress and initiate the UPR to restore normal ER function. If the stress is prolonged, or the adaptive response fails, apoptotic cell death occurs (12).

As a result of ER stress, cells mainly develop two responses: one leads to cell survival and the other leads to apoptosis (13). Using the survival pathway, cells overcome such adverse effects and maintain homeostasis through the UPR, from They inhibit mRNA transcription, increase the folding capacity of ER and Endoplasmic-reticulum-associated protein degradation (ERAD) to restore homeostasis (14). Under chronic or severe ER stress, the normal functions of ER are not recovered, resulting in cell dysfunction and apoptosis (14) Therefore, the ER is considered a quality control checkpoint and only correctly folded proteins can exit the ER and pass through the secretory pathway. Therefore, any event such as starvation and excessive protein synthesis, accumulation of mutant proteins, depletion of ER calcium, or changes in the redox state that disrupt the folding capacity of the ER triggers a physiological response called the unfolded protein response (UPR). These homeostatic responses cause the production of additional chaperones to increase the folding capacity of ER, increase protein degradation related to the endoplasmic reticulum, and by changing the translation and synthesis of new proteins, reducing protein entry and thus organelle and cell balance (15). Studies show that silencing C/EBP homologous protein (CHOP) reduces liver apoptosis in alcohol-induced diseases and cholestasis-induced fibrosis (16).

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CHOP can also regulate the expression of autophagy-related genes in the later stages of starvation, CHOP can prevent the occurrence of autophagy and soon initiate apoptosis (17), however, the role of CHOP in NAFLD is debatable (18). One study found that CHOP can prevent it (19,20). Also, experiments on mice with CHOP show that CHOP is associated with many diseases that cause ER stress (21).

Chronic ER stress interferes with body metabolism by activating lipogenesis and increasing VLDL (22). Research is ongoing to provide alternative non-pharmacological pathways to reduce the risks of NAFDL. Exercise is one of the ways to replace drugs in this disease for any disease, Hutton et al. (23,24) also, Bucky et al. (25) suggested that both aerobic and resistance exercises have similar effects on liver TG in patients with NAFLD. These studies show that different types of exercise help reduce NAFLD. Also, interventional studies have shown that regular exercise can reverse ER disorders (26) and UPR activation has been shown to reduce ER stress (27,28). The UPR is an important mechanism for modulating fatty acid oxidation and lipogenesis (29). Furthermore, chronic fasting conditions in mice have been shown to activate the UPR to regulate lipid metabolism (30). Studies have shown that XBP1 regulates genes involved in various cellular processes, such as ER stress response, secretory function, lipid metabolism, glucose homeostasis, and inflammation (31,32). XBP1 regulates the expression of genes involved in fatty acid synthesis and increases hepatic lipogenesis (33). Several studies have shown that XBP1 plays an important role in adipocyte differentiation by regulating morphological and functional changes during adipogenesis (34). The importance of XBP1s in lipid biosynthesis has been demonstrated. It causes triglyceride (TG) biosynthesis and abnormal fat accumulation (35).Chronic starvation in mice has been shown to activate the UPR to regulate lipid metabolism (36). Also, studies show that exercise up-regulates hepatic XBP1 and SREBP through ERS signaling, thereby reducing lipid accumulation in NAFLD liver (37). The contradictions from human and animal experiments led us to investigate the effect of aerobic running on a treadmill and 4-week starvation in regulating the activity of the apoptotic pathway caused by endoplasmic reticulum stress in the liver of male rats with non-alcoholic fatty liver disease.

Methods

30 obese male Wistar rats aged 18-20 weeks with an average body weight of 348 ± 25.53 after one week familiarization with the laboratory environment were randomly divided into 6 groups of five, 1: starvation group, 2 and 3: 3 and 5 training days respectively), 4 and 5: (groups 3 and 5 training days with starvation) and 6: control group were divided

All fatty model animals had free access to water and standard pellet food (10 g of food per 100 g of mouse body weight). All maintenance and sacrifice procedures were carried out in the Animal Science Laboratory of Gorgan Medical Sciences. The fasting protocol was applied for one month and every day for 14 hours in the waking cycle (5.5 pm to 5.7 am). In order to induce hunger, the rats in the starvation group were given the same amount of food (10 grams per 100 grams of mouse weight). They received the same food over 10 hours as the other groups received over 24 hours.

The entire training course includes two stages of familiarization and main training. For this purpose, perform the test conditions for 15 minutes for a week and exercise for 45-60 minutes on the treadmill, 3 and 5 days a week for 4 weeks. The training of the rats started on a treadmill with

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a 0-degree incline. With a speed of 14 meters and after finishing the training sessions, the speed of the treadmill with zero incline reached 16 and 18 meters per minute (38).
Biochemical factors of HDL were measured by enzymatic method and LDL and VLDL by calorimetric method, respectively, using the biochemical kits of Darman Kav and Far Samad manufactured in Iran and BS480 Auto analyzer.
Finally, the ratios of VLDL/HDL and LDL/HDL were calculated for statistical analysis. For molecular investigations at the level of gene expression, RNA was first extracted from the tissues in all investigated groups, according to the protocol of Yekta azma Equipment manufacturer (cat.No:FABRK001 lot.No:K812320822). Then, we measured the quality and quantity of RNA with the Nanodrop device of Golestan University of Medical Sciences and analyzed it with the cDNA synthesis assay kit of Pars Tous Company of Mashhad (parstous.lot:2156, REF: A101161) and then the synthesized cDNA was used to perform the reverse transcription reaction. Was used Expression levels of xbp1 chop genes were measured using real-time steps quantitative method. The primers were made by SYBER Green qPCR master mix (cat.No: YT2552, lot.P2003), and the primers were ordered by Pishgam Biotech company. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a control gene and the expression level of the desired gene was calculated with the formula 2-ΔΔCT in the following way. First, the threshold cycle of the desired gene of each sample was calculated from the threshold cycle of the homeostasis gene. The sample was subtracted. (ΔCt=Ct Target-Ct Housekeeping) In the next step, the number obtained from delta Ct of each sample was subtracted from the samples to which it needed to be compared (ΔΔCt=ΔCt Target-ΔCt Reference) and we multiply the negative of the obtained number to the power of two Target gene/Reference gene ratio = 2 -ΔΔCT and
We obtain the relative expression of xbp1 chop gene .The primers used are reported in Table 1below. The size of the genes is as follows C/EBP homologous protein gene ID: DDIT3 gene length 150 bp and X-box binding protein 1 gene ID: XBP1 gene length: 601 bp.

Table 1: Sequence of primers used

Genes	Primer sequence (5' → 3')	Number of nucleotides	Amplicon Size(pb)
Chop-F	GAAAGCAGAAACCGGTCCAAT	21	**150**
Chop-R	GGATGAGATATAGGTGCCCCC	21	-
XBP1-F	AAACAGAGTAGCAGCGCAGACTGC	24	**601**
XBP1-R	GGATCTCTAAAACTAGAGGCTTGGTG	26	-
GAPDH-F	CACTGAGCATCTCCCTC ACAA	22	-
GAPDH-R	TGGTATTCGAGAGA AGGGAGG	22	-

In order to check the descriptive statistics of the mean and standard deviation and estimate the inferential statistics from the one-way analysis of variance (p) and the LSD follow-up test among the research groups, it was shown in Tables 3 and 4, respectively.

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Result:

The results obtained from Table (2) and the average of Graph 1(A,B) showed the lowest mean LDL/HDL ratio in the 5-day training plus fasting group (0.28±0.61) and the lowest VLDL/HDL value was also shown in the 5-day training plus fasting group (0.12±0.286), which indicates the better effects of combined training and fasting. Also, the lowest mean XBP1 gene expression (0.13±0.20) was seen in the fasting plus 5-day training group and a greater decrease in the mean chaperone gene expression (0.06±0.15) was seen in the 5-day training plus fasting group. Genes involved in the inflammatory pathway promote autophagy in non-alcoholic fatty liver patients with exercise and fasting.

The results of one-way analysis of variance in Table 3 show a significant change in the ratios of LDL/HDL (P=0.00, F=23.986) and VLDL/HDL (P=0.00, F=23.986), as well as the expression of the genes CHOP (P=0.00, F=23.986) and XBP1 (P=0.00, F=23.986). Also, the LSD follow-up test showed a significant decrease in the values of VLDL/HDL, LDL/HDL (in all experimental groups compared to the control group (P = 0.00) and also a significant decrease in the expression of XBP1 genes in the 5-day training groups and the 3 and 5-day training with starvation groups (P = 0.00), but there was no significant change in the starvation group and the 3-day training alone group (P= 0.845 and P = 0.055, respectively). The chaperone gene also showed a significant decrease in all groups except the starvation alone group (P = 0.580) compared to the control group (P = 0.00).

Table 2: Mean and standard deviation of fatty model rats

mean and standard deviation of variables	starvation group + 5 days of training	starvation group + 3days of training	5days training group	3days training group	starvation group	control group
VLDL/HDL	0.286±0.12	0.72±0.31	0.69±0.21	0.94±0.70	0.73±0.21	2.43±0.43
LDL/HDL	0.61±0.28	1.23±0.32	1.03±0.24	1.17±0.69	1.79±0.32	3.69±0.81
CHOP	0.13±0.20	0.22±0.22	0.30±0.23	0.55±0.38	1.08±0.15	1.00±0.00
XBP1	0.06±0.15	0.22±019	0.35±0.31	0.77±0.21	0.97±0.43	1.00±0.00

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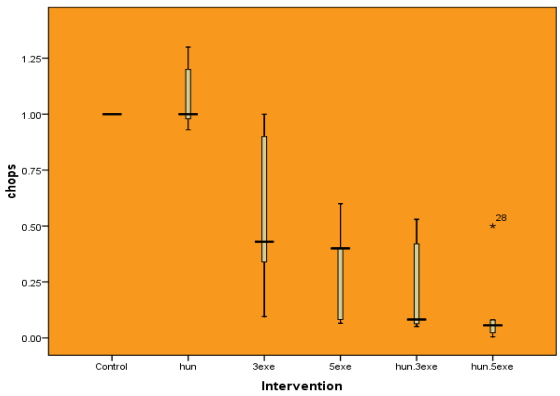
Figure 1: **A** **B**



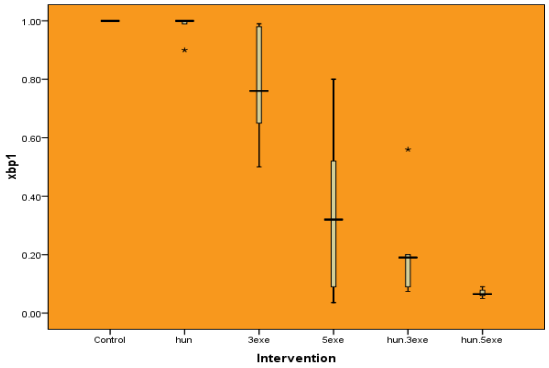


Table 3: One-way analysis of variance test results to compare the means between groups

Variables	Sources of change	sum of squares	mean square	df	f	p
LDL/HDL **Mg/dl**	Intergroup	140.084	60.39	5	23.986	.000
	within group	6.403	0.252	24		
	total sum	36.240		29		
VLDL/HDL **Mg/dl**	Intergroup	14.084	2.817	5	19.269	.000
	within group	3.509	.146	24		
	total sum	17.593		29		
CHOP **Ng/mol**	Intergroup	4.103	.821	5	15.340	.000
	within group	1.284	.053	24		
	total sum	5.386		29		
XBP1 **Ng/mol**	Intergroup	4.063	.813	5	26.290	.000
	within group	0.742	.031	24		
	total sum	4.805	60.39	29		

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Discussion

The aim of the study was the effect of aerobic exercise running on a treadmill and starvation for 4 weeks on the regulation of the apoptotic pathway activity caused by endoplasmic reticulum stress in the liver of male Wistar non-alcoholic fatty model rats. The results of XBP1 description of this research showed that the highest average was observed in the starvation group and the lowest average was observed in the (fat + starvation + 5 days of training) group. The results of the research showed that performing 4 weeks of aerobic exercise along with starvation reduces the expression of both genes involved in the development of NAFLD. While the control and starvation groups have the highest average expression of these genes, exercise decreases the expression. A review of research shows that exercise controls the transcription of XBP1 in the liver (35,36) . Various mechanisms can be involved in this, such as previous studies showing that the goal of starting the UPR is to restore homeostasis and normal ER function. And adaptive mechanisms that increase the expression of genes involved in increasing the capacity to eat ER protein (35). When the primary stimuli that cause UPR not to be eaten are long or excessive, UPR adaptive mechanisms fail. And cell death occurs through apoptosis (37).

It has been reported in different studies that starvation causes a decrease in nutrients inside the cell and it's sensing by brain material-sensing signaling pathways such as mTOR and AMPK pathways, which ultimately stimulates autophagy (39). In addition, p-eiF2α selectively promotes the translation of an increasing number of mRNAs, including ATF4. He does. Activation of IRE1 causes modification of XBP1 and subsequent transcription of molecular chaperones and genesinvolved in ERAD (40). Finally, activated ATF6 undergoes proteolytic cleavage in the Golgi, allowing its mature form to enter the nucleus and ER stress-related genes. Such as ER chaperones and foldases (41) .The findings show that starvation activates the IRE1α-XBP1 signal (42). The findings from previous studies show that the combination of fasting diet, acute resistance training and Protein consumption (immediately or 1 hour after exercise stimulation) increases the serum levels of leucine, insulin and glucose, as well as the content of autophagic protein in skeletal muscles (43,44) .but it reduces other proteins related to the autophagic pathway in the liver (45) . It has also been shown that 6 weeks of wheel running suppressed XBP1s mRNA increase in HFD-fed mice, and similar results were shown in mice after 6 weeks of treadmill training (45). In addition, swimming exercise decreased IRE-1α and XBP1 protein levels and decreased hepatic TG content in rats with NAFLD. (47) Lu et al showed that exercise decreased SREBP-1 induced fat accumulation. In the liver through the AMPK pathway to inhibit the mammalian target of rapamycin complex 1 and relieve ERS (46). Overall, exercise reduced hepatic lipogenesis via the PERK/ATF4/SREBP pathway (44,46) . These studies show that exercise regulates hepatic XBP1 and SREBPs through ERS signaling and thus reduces fat accumulation in the liver of NAFLD. Exercise helps reduce excessive aberrant phosphorylation in the endoplasmic reticulum that causes apoptosis and cell death. Also, endurance activity such as swimming causes that due to the compatibility between these transmembrane proteins, the amount of misfolded or over folded proteins in the endoplasmic reticulum, which cause stress, decreases. As a result, the amount of stress in the endoplasmic reticulum decreases. Of course, the results of some studies are not consistent with the present study. Among other things, in a research where short-term sports activity such as a one-day sprint or a five-day activity for a week has no effect on the expression of XBP1, ATF6, and PERK proteins, which is probably due to the duration of the sports activity

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(43, 45) . Because the duration of their activity is less than a week. If it has been shown to express these proteins, the minimum time of sports training should be four weeks. It has also been reported that rats that had a history of sports activity had less stress symptoms and the expression of UPR (XBP1, ATF6) and CHOP genes after resistance training than rats that did not have any sports activity, which shows that Sports activity has a positive effect on reducing stress symptoms in exercise rats (49). Since sports activity is a therapeutic solution to reduce liver diseases including NAFLD. The results of the present study showed that NAFLD increases the expression of XBP1 and CHOP genes, and this increase was significant in the control group compared to the exercise and exercise + starvation groups, which can be a possible confirmation that endoplasmic reticulum stress is one of the One of the main causes of cell apoptosis in the liver. According to the statistical results of our research, it showed a significant decrease in the ratio of lipoproteins (VLDL/HDL, LDL/HDL) in all groups compared to the control group. In recent years, several clinical trials have shown that starvation is an effective way to reduce fat and regulate lipid profile. Starvation or energy-restricted diets have favorable effects on body weight, total fat mass, and liver fat reduction. In addition, intermittent fasting can improve biomarkers of systemic inflammation and appetite-regulating hormones (50). A recent finding suggests that exercise is better than a calorie restriction program in cholesterol biosynthesis. Short-term exercise combined with dietary interventions has a great effect on reducing metabolic risks and fasting insulin levels (50). In a study by Askari et al. in 2012, they showed that 8 weeks of aerobic exercise reduced the percentage of subcutaneous fat, total cholesterol, RF and plasma low-density lipoproteins in non-athletic women (51) Also, in another study by Dadban et al. in 2021, it was shown that 4 weeks of regular aerobic exercise reduced liver lipase and thus reduced triglyceride production in VLD L-C and LDL-C. Elevated LDL-C is an independent risk factor for coronary artery disease, while lowering LDL-C to 60 mg/dL reduces the risk of coronary heart disease by 50% within two years. HDL-C transports cholesterol from peripheral tissues to the liver and then directs excess cholesterol to the bile for excretion (52). However, our results are consistent with the findings of the aforementioned studies. In summary, in such research, understanding the effect of diet on disease severity is one of the most complex aspects in the management of patients with nonalcoholic fatty liver disease. And as a result, evaluating the effect of dietary interventions is challenging because it affects the entire metabolism and it is difficult to isolate specific (beneficial) effects on the liver. However, a low-calorie, low-carbohydrate diet combined with continuous aerobic exercise with repetitions of at least 3 days and up to 5 days of exercise could potentially be suitable for the successful "treatment" of NAFLD.

Conclusion

Impaired autophagy may be a critical mechanism in the pathogenesis of nonalcoholic fatty liver disease, and the role of exercise and starvation as an important tool in the prevention of nonalcoholic fatty liver disease. The findings of the research showed that the breakdown of lipids in the liver moderated the disease due to the increase in non-selective autophagy of the liver endoplasmic reticulum. Running with starvation helps NAFLD rats to control the level of CHOP and XBP1 genes and help apoptosis and removal of waste cells as well as reduce ER stress, so this method can be used as a safe and healthy interventional treatment in the treatment of the disease. Liver diseases (NASH, NAFLD) should be considered.

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Ethical statement

This study was conducted using the research ethics code previously approved by Golestan University of Medical Sciences with the ID. IR.GOUMS.REC.1401.005.

Conflicts of interest

There is no conflict and interest by the authors.

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