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Original Article

The effect of 12 weeks of aerobic exercise and caloric restriction on Nrf2 protein expression in non-alcoholic fatty liver disease in rats

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Abstract

Background and aims: One of the most common causes of liver disease is non-alcoholic fatty liver disease (NAFLD), and its prevalence along with serious clinical problem is a growing. No studies have been conducted on the effect of calorie restriction (CR) and aerobic exercise (AE) on nuclear factor erythroid-related factor 2 (Nrf2) in rats with NAFLD. The present study aimed to assess the effects of 12 weeks of CR and AE on Nrf2 protein expression in rats with NAFLD.

Methods: A total of 40 rats participated in this study, and 32 of them developed NAFLD by feeding fatty food (34% fat, 19% protein, and 47% carbohydrates) for eight weeks. Rats were classified into five groups: sham, control, CR, AE, and calorie restriction-aerobic exercise (CA). First, 60% of the daily diet was given to the CR and CA groups. AE was done for 12 weeks, five sessions per week on a treadmill for rats. Oil red, hematoxylin-eosin (H & E) staining, and protein expression levels in the groups were evaluated. To analyze the data, one-way ANOVA was used at a significance level of $P<0.05$.

Results: The results showed a significant difference between the liver fat of the control group and other groups (RC: $P=0.001$, AE: $P=0.001$, RA: $P=0.001$). In healing liver damage, the control group was significantly different from the CA group ($P=0.002$). Regarding the Nrf2 protein expression, the CA group had significantly higher expression than the CR group ($P=0.028$), however there was no significant difference between the CA and AE groups ($P=0.44$).

Conclusion: The findings revealed that AE through CR can cause recovery for NAFLD.

Keywords: Non-alcoholic fatty liver disease, Nrf2, Caloric restriction, Aerobic exercise

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease affecting nearly 30% of the people in the world (1). In the initial phases of NAFLD, the periphery incremented liver fat load makes the production of reactive oxygen species implicated (1). Nuclear factor erythroid-related factor 2 (Nrf2) as a well-known master regulator of redox homeostasis is a major transcription factor included in the defense against oxidative stress (1). Nrf2 induces the production of major anti-inflammatory changes, stimulates the emergence of mitochondrial life, improves mitochondrial function, and stimulates autophagy; further, it causes the destruction of seeds, the toxicity of the protein, and inefficiency of organelles (2). To develop steatohepatitis, Nrf2 is a considerable regulator of the redox balance mediating the antiapoptotic and anti-inflammatory effects of antioxidants (3). In addition, lipid metabolism is directly affected by Nrf2 via activating the genes included in triglyceride export (apolipoprotein B), fatty acid oxidation (acyl-CoA oxidase 2 and carnitine palmitoyltransferase 1), and the lipogenic transcription factor sterol regulatory element-binding protein 1 (4). Studies in which mice received a high-fat diet (HFD) have also reported that hepatic lipogenesis is negatively regulated by Nrf2 (5). According to these data, changing the antioxidant pathways and metabolism are related to NAFLD (3).

Calorie restriction (CR) is one of the most effective interventions for treating NAFLD, and it is postulated that recognizing the involved mechanisms provides accurate knowledge for finding the most effective bioactive or pharmacological interventions (6). Respiration rates are stimulated by CR in mammals, increase mitochondrial density and biogenesis in tissues, and decrease the coupling between oxidative phosphorylation and oxygen uptake (7). Generally, major metabolic reprogramming is caused by CR to reduce oxidative damage to macromolecules and the effective utilization of fuel (8). So far, the direct effect of CR on Nrf2 expression has not been investigated.

Studies have indicated that exercise activity by increasing Nrf2, heme oxygenase-1 (HO-1), and superoxide dismutase decreases oxidative stress (9). Several lines of studies also showed that aerobic exercise (AE) significantly reduces hepatic fat content by 3-40%

(10). AE activity appears to reduce fatty liver damage by increasing Nrf2 expression through controlling oxidative stress and improving inflammatory status (11). Researchers have concluded that regular physical activity enhances the antioxidant defense system due to its adaptation to oxidative stress (2). Many studies have demonstrated that an active lifestyle improves Nrf2 function. In contrast, a sedentary lifestyle has negative effects, significantly reducing nuclear Nrf2 expression. Further, due to the compatibility of endurance training, Nrf2 protein enhances respiratory capacity and increments adenosine triphosphate production during exercise (2). Due to the limited evidence of the effects of CR and AE on Nrf2 protein expression in NAFLD and the effect that AE and CR can have on the prevention and treatment of NAFLD through Nrf2 expression, this study investigated the effect of CR and AE on Nrf2 protein expression in rats with NAFLD.

Materials and Methods

Animals

For this experiment, 40 two-month-old male Wistar rats were bought from the Pasteur Institute of Iran. The animals were kept under new conditions for two weeks with free access to food and water; thus, stress was prevented and physiological conditions were changed. The animals tested in this study were kept in transparent polycarbonate cages in pairs at a humidity of 40% to 60% and a temperature of 22 ± 3°C on a light and dark cycle 12:12. According to the instructions of the Committee for Work with Laboratory Animals, while observing ethical issues, any physical harm and unnecessary methods of working with animals were avoided in various stages of the study, and rats were weighed once a week on a specific day. To develop fatty liver after two weeks, 32 rats were randomly placed in the HFD group and eight rats in the normal diet group (sham). After induction of fatty liver with the regime (34% fat, 19% protein, and 47% carbohydrate) for eight weeks (12), 32 rats that ate fatty food were divided into four groups, and eight rats that ate normal food were named the sham group. Groups include high-fat control, sham, AE, CR, and CA. Then, 60% of the daily diet (8) was given to the CR and CA groups. AE and CA groups performed an AE program on an animal-smart electronic treadmill for 12 weeks (5 days a week). The relative intensity of work throughout the exercise program 24-33 m/min was maintained with a slope of 15%. Training duration of 10 minutes a day started in the first week and approached 60 minutes in the fifth week (13).

To determine the amount of consumed food and CR, the amount of food consumed daily by rats was measured for two weeks, and in groups with limitations, food was 60% of food consumed by healthy rats. Then, 48 hours after the last training session (fasting for 12-14 hours), rats became unconscious by intraperitoneal injection of a combination of 60 mg/kg ketamine and 5 mg/kg xylazine and then underwent surgery (14).

After surgery, the lower lobe of rat liver tissue was cut and immediately collected and frozen at -80°C. Then, it was stained with oil red and hematoxylin-eosin (H & E), and another part was used for analysis and counting of Nrf2 protein by Western blot.

Western blot analysis

To extract liver tissue Nrf2 protein from radioimmunoprecipitation buffer containing 0.05 mM Tris buffer (pH 8), 150 mL sodium chloride molar, 0.01% EGTA, 1% sodium dodecyl sulfate, and 0.1% cocktail anti-protease (ROCHE) was used. For protein extraction, 100 mg of tissue was homogenized by a hand homogenizer in 500 µL buffer containing anti-protease and was left for half an hour. The temperature was set at 4°C, and then a centrifuge refrigerated (bo, SW14R froil) was centrifuged at 12000 rpm and 4°C for 10 minutes. Next, the supernatant was gathered, and its protein concentration was measured by a Bio-Rad protein determination kit at 595 nm. Finally, it was kept at -20°C and then homogenized in a 1:1 ratio with sample loading buffer (50 mM Tris-chloride hydrogen, 2% sodium dodecyl sulfate, 5% beta-mercaptoethanol, 10% glycerol, and 0.005% aqueous bromophenol) Then, the samples were boiled for 5 minutes until all the proteins were fully denatured. Proteins were isolated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane. The membrane was blocked for one hour in 5% bovine serum albumin in 0.1% Tris Buffered Saline with Tween 20 and incubated in the initial antibody (1: 500). I t was incubated in the secondary antibody the next day for one hour at room temperature in 4% TBST. Proteins with one electrochemiluminescence reaction were measured by densitometry analysis ImageJ software. Primary and secondary antibodies were antibody cat number: Nrf2: orb224910 and rabbit number: BA1054-2, respectively.

Histopathological analysis

Oil red analysis

Frozen incisions were made in sizes of 8 to 10 microns, and then the sections were dried in the open air. The slides were subsequently fixed in formalin (Sigma -1.04002) and could be briefly flushed with tap water. The slides were installed with 60% isopropanol (Sigma-I9516) and placed in Oil red working solution for 15 minutes. The samples were then washed with 60% isopropanol, and the nuclei were stained with hematoxylin (Sigma-H9627). Next, they were washed with distilled water, and glycerin gel was used to assemble the slides. Afterward, photography was done with a light microscope (LABOMED).

H & E analysis

First, the slides were placed in incubator at 90°C for 20 minutes to melt the paraffin in the sample. In the second stage of paraffinization, the samples were placed inside xylol 1 and 2 (730-1330-Sigma) for 15 minutes. Finally,

a drop of Entellan glue (Sigma-1.07961) was placed on the sample, then the lamellar was glued to the slide, and photography was taken with a light microscope (LABOMED).

Statistical methods

The mean and standard deviation were used for reporting descriptive data. After checking the normality of the data with the Shapiro-Wilk test for the comparison between groups of variables, if the distribution of data was normal, the one-way ANOVA at the significance level of $P<0.05$ and Tukey's follow-up test would be used.

Results

CR and CA reduced body weight and liver weight. Body weight in the control group showed a significant change only before the protocol and 11 weeks after the protocol ($P=0.045$), and no significant difference was observed between the sham group ($P=0.89$); similarly, no significant intra-group difference was observed in the AE group ($P=0.737$). A significant change in body weight was observed in the CR group ($P=0.001$). Moreover, the change of body weight in the CA group in weeks 8 ($P=0.008$), 11 ($P=0.002$), and 12 ($P=0.001$) after the protocol was statistically different compared to the time before the protocol (Table 1).

A significant difference was found in liver weight between the CR group and the sham ($P=0.001$), control ($P=0.001$), and AE ($P=0.001$) groups. However, no significant differences were found between the CR group and CA ($p=0.363$) and also between the AE group and the control group ($P=0.572$). In the AE group, liver weight was significantly higher than CR ($P=0.001$) and CA ($P=0.001$) groups (Figure 1).

CR, AE, and CA ameliorated hepatic histopathological changes induced by HFD. Table 2 depicts the weighted mean, standard deviation, and significant difference between the groups. Microvesicular steatosis was revealed by histological observations in the model rats' livers as confirmed by excessive small lipid droplets within the hepatocytes swelling and cytoplasm (15). Furthermore, 5% of nucleated hepatocytes were observed with an apoptotic appearance. A normal histological structure was also found in the livers of the sham group with no inflammation and steatosis. In addition, a significant difference was observed between the liver fat of the control group and other groups (CR: $P=0.001$, AE: $P=0.001$, and RA: $P=0.001$). Likewise, there was a significant difference in liver fat between CA and CR ($P=0.001$) and between CA and AE ($P=0.001$) groups, and there was no difference in liver fat between CR and AE groups ($P=0.26$). Compared to the control group, the rat livers' histological structure enhanced in the other groups, but in the control group, due to inflammation and the destruction of part of the liver tissue, dead cells with less staining were observed rather than living cells, which

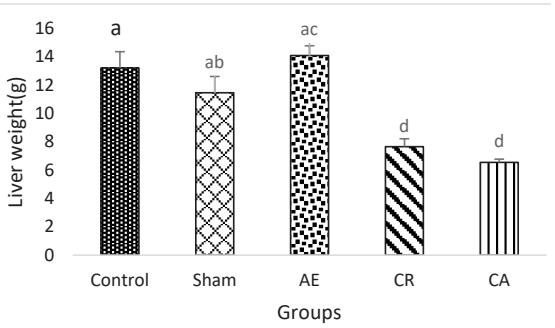

Figure 1. CR and CA reduces liver weight. *Note.* AE: Aerobic exercise; CR: Caloric restriction; CA: Caloric restriction-aerobic exercise. Dissimilar acronyms indicate significant differences.

Table 1. Comparison of body weight at the baseline and study interval between the AE, CR, and CA groups

Weeks	Groups									
	Control		Sham		AE		CR		CA	
	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value
0	269.33 ± 54.60		313.66 ± 22.94		360.66 ± 27.09		320.00 ± 26.08		347.66 ± 11.23	
1	324.66 ± 62.85	0.934	315.66 ± 22.94	1.00	363.33 ± 26.0	1.00	301 ± 3.60	0.001*	325.33 ± 21.59	0.958
2	355 ± 43.85	0.489	309.66 ± 24.66	1.00	368 ± 26.85	1.00	286.66 ± 2.30	0.001*	312 ± 15.58	0.943
3	366 ± 42.03	0.317	328 ± 18.24	1.00	374.33 ± 26.63	1.00	289.66 ± 3.05	0.001*	315.66 ± 19.85	1.00
4	349.66 ± 41.13	0.583	326 ± 21.93	1.00	370.66 ± 25.57	1.00	285.66 ± 0.57	0.001*	314.66 ± 9.07	0.998
5	375.66 ± 41.64	0.201	340.66 ± 24.94	0.971	377.66 ± 25.42	1.00	303.33 ± 4.04	0.002*	317.33 ± 15.56	1.00
6	369.33 ± 37.07	0.273	342.66 ± 27.42	0.952	369.33 ± 23.07	1.00	299.33 ± 3.21	0.001*	325.66 ± 19.60	1.00
7	377.33 ± 34.12	0.185	341.33 ± 24.44	0.965	348.66 ± 55.77	1.00	308 ± 4.58	0.076	345 ± 15.09	1.00
8	374.33 ± 43.14	0.215	326.66 ± 27.53	1.00	374.33 ± 19.39	1.00	299.33 ± 4.04	0.001*	279.33 ± 11.15	0.008*
9	400 ± 41.21	0.051	354 ± 24.51	0.702	389 ± 18.35	0.981	300.66 ± 3.05	0.001*	283.33 ± 3.05	0.131
10	400 ± 46.8	0.051	362.33 ± 2542	0.439	392.66 ± 16.28	0.955	312.33 ± 9.01	0.479	302 ± 2.64	0.811
11	402 ± 43.58	0.045*	369 ± 27.02	0.263	393.33 ± 20.23	0.948	288.33 ± 8.50	0.001*	273.66 ± 12.42	0.002*
12	393 ± 34.21	0.078	355 ± 21.64	0.671	383.33 ± 17.55	0.997	263.33 ± 3.78	0.001*	244.33 ± 11.59	0.001*

Note. AE: Aerobic exercise; CR: Caloric restriction; CA: Caloric restriction-aerobic exercise; SD: Standard deviation.
* Significance at $P<0.05$ level.

Table 2. Comparison of liver fat, inflammation, damage, and relative expression of Nrf2 protein between the AE, CR, and CA groups

Variable	Groups	Mean ± SD	The difference between the control and other groups P value	The difference between the Sham and other groups P-value	The difference between the AE and other groups P value	The difference between the CR and other groups P value	The difference between the CA and other groups P value
Liver fat	Control	63.35 ± 1.606		0.001*	0.001*	0.001*	0.001*
	Sham	11.24 ± 2.570	0.001*		0.001*	0.001*	0.394
	AE	31.94 ± 5.567	0.001*	0.001*		0.260	0.001*
	CR	36.19 ± 3.234	0.001*	0.001*	0.260		0.001*
	CA	14.86 ± 1.468	0.001*	0.394	0.001*	0.001*	
Inflammation and liver damage	Control	1.333 ± 0.2887		0.001*	0.061	0.061	0.002*
	Sham	0.00 ± 000	0.001*		0.061	0.061	0.928
	AE	0.6667 ± 0.2887	0.061	0.061		1.00	0.20
	CR	0.6667 ± 0.2887	0.061	0.061	1.00		0.20
	CA	0.1667 ± 0.2887	0.002*	0.928	0.20	0.20	
Nrf2	Control	0.2239 ± 0.0579		0.001*	0.004*	0.037*	0.002*
	Sham	1.336 ± 0.1384	0.001*		0.023*	0.003*	0.130
	AE	0.8830 ± 0.0564	0.004*	0.023*		0.163	0.442
	CR	0.6270 ± 0.0566	0.037*	0.003*	0.163		0.028*
	CA	1.055 ± 0.1126	0.002*	0.130	0.442	0.028*	

Note. Nrf2: Nuclear factor erythroid-related factor 2; AE: Aerobic exercise; CR: Caloric restriction; CA: Caloric restriction-aerobic exercise; SD: Standard deviation.
* Significance at $P<0.05$ level.

was due to the lack of tissue staining. Unlike the control group, the severity of hepatic steatosis significantly improved only in the CA group ($P=0.002$), as indicated in Table 2. No significant difference was found in terms of improvement and inflammation between the control group and the CR ($P=0.061$) and AE ($P=0.061$) groups as well as between the CR and AE groups ($P=1.00$). Moreover, no significant differences were observed between the CR ($P=0.2$) and AE ($P=0.2$) groups and the CA group in terms of the improvement in the severity of steatosis and inflammation.

As shown in Table 2, Nrf2 protein expression increased significantly between the CR ($P=0.037$) and AE ($P=0.004$) groups and CA ($P=0.002$) group compared to the control group. However, no significant differences were found in Nrf2 protein expression between the CR and AE groups ($P=0.163$). Protein expression in the CA group was higher significantly than in the CR group ($P=0.028$), but no significant differences were observed between AE and CA groups ($P=0.442$).

Discussion

Over the last twenty years, NAFLD has become doubled. Currently, there are no available effective drugs for NAFLD management in the clinics mainly owing to a lack of information on the underlying mechanisms of the disease (1). In the current study, we developed a NAFLD rat model by feeding rats an HFD for eight weeks. Then, after grouping, they were subjected to the CR and AE for 12 weeks. Body weight increased in the control group. CR resulted in weight loss in rats with nonalcoholic fatty liver, and studies indicated that weight loss improves NAFLD. Decreasing the excess body weight by 5%-10% may improve liver histology, fibrosis, and serology declarative of liver damage in NAFLD patients (16). Moreover, according to recent observations, regular exercises such as aerobic activities, resistance exercise, and flexibility training improve NAFLD independently of weight loss. It also improves systemic markers of liver function and intrahepatic fat in mild-to-advanced NAFLD (10). In the AE group, liver weight was significantly higher than that in CR and CA groups, which seems to be due to the increase in muscle and liver glycogen capacity.

As displayed by Oil red, liver lipid levels were higher significantly in the control group than in the other groups, which caused inflammation and damage in this group. The AE and CR reduced liver fat. In the present study, it was observed that 12 weeks of AE improved liver fat. in the same vein, Oh et al indicated that 12 weeks of moderate-intensity continuous cycling or vigorous-intensity interval cycling causes significant reductions in intrahepatic fat content (17). Several studies also indicated that resistance training reduces hepatic fat content independently of weight loss (10). Shojaee-Moradie et al observed a significant reduction in intrahepatocellular fat in NAFLD patients who exercised a combination of aerobic and resistance training (18). Additionally, research showed that reducing fat accumulation and maintaining lipid homeostasis that occurs by swimming exercise can be a positive response of zebrafish liver to exercise (19). Another study revealed that CR prevents the accumulation of glycerolipids in the liver of rats under CR, and they are degraded to FFAs by increased autophagy (8).

H & E in the present study showed that caloric restriction and AE simultaneously can improve the effects

of inflammation and tissue damage. The amount of inflammation and damage in both CR and AE groups was also reduced separately, though it was not a significant amount. One study indicated that obesity causes intrahepatic lipid oxidative damage, and weight loss could reduce it to levels similar to normal people (20).

The progression of NAFLD may be not prevented by adaptive Nrf2 activation induced only by high-fat feeding in the absence of an additional Nrf2 activator. Thus, Nrf2 expression pharmacological activation can potentially attenuate the fatty liver disease onset (21). In this study, the effect of CR and AE on Nrf2 protein expression in rats with non-alcoholic fatty liver was investigated. It was found that the expression of Nrf2 protein in the control group was significantly lower than that in the other groups, which is in line with studies showing that the inability to adapt to hepatic oxidative stress may accelerate the NAFLD development in Nrf2 deficient mice (13). NAFLD is a class of chronic disorders, including oxidative damage, antioxidant and inflammatory responses, impaired lipid metabolism, inflammation, and fibrosis. In such pathophysiologic events, Nrf2 is investigated widely as a potential therapeutic target of the disorders. Several studies performed on Nrf2 transgenic mice and Nrf2 activators revealed that lipid metabolism can be improved by Nrf2 activation, and the liver is protected from inflammation and oxidative stress (14).

The findings of this study demonstrated that CR causes the expression of the Nrf2 protein. Under the CR conditions, peroxisome proliferator-activated receptor-γ coactivator 1-α is expressed and activated, leading to an increase in mitochondrial biogenesis through the activity of coactivators such as Nrf1 and Nrf2 (22). It seems that in this way it improves the function of metabolism and the antioxidant system as well as NAFLD. In addition to the earlier revealed paths regulating mitochondrial activity against the CR, the effect of polyphenols and CR is also accompanied by Nrf2 higher activity (23). Through this transcription factor, the gene's plethora of expression is regulated, detoxifying electrophiles and oxidants, repairing or removing damaged macromolecules, and reducing inflammation and oxidative stress related to the damaged mitochondria accumulation (22). Furthermore, a reduction was found in the expression of Nrf2 protein in the control group, which could indicate a decrease in antioxidant activity due to HFD in this group. Additionally, liver mRNA levels of Nrf2 and its downstream targets decreased in obese animals fed an HFD (24). This is in line with the results obtained in the present study. It is important that the suppression of Nrf2 induced by HFD be reversed with CR and AE similar to the present work.

The endogenous antioxidant defense systems are induced by the physical exercise-generated oxidative stress that themselves are regulated by Nrf2, to a great extent (25,26). Studies have indicated that exercise reduces oxidative stress by increasing Nrf2, HO-1, and superoxide dismutase. Nrf2-related signaling pathways appear to have an antioxidant effect (27). Regular AE has been shown to play a key role in preventing cellular oxidative damage by increasing the activity of the HO-1/Keap-1/Nrf2 pathway in the liver, kidneys, and heart (28). Wafi et al showed that exercise regulates Nrf2 expression and antioxidant levels in mice (27). In the present study, we found that AE for 5 days a week increased Nrf2 protein expression in rats with NAFLD. Consistent with our study, in a study, two consecutive days of treadmill running (60 minutes per day; 14m/min, 10% grade) improved the function of Nrf2 to protect the oxidative stress in mouse cardiac muscle in wild-type and Nrf2-/-mice (29). Likewise, another study revealed that physical exercises for six weeks with moderate intensity can dramatically increase the expression of the Nrf2 protein nucleus in the heart of young and old rats, which can be concluded that moderate exercise increases the expression of Nrf2 (30). In addition, one study reported that five weeks of moderate-intensity endurance training improves antioxidant defense in young men, and another study demonstrated that acute AE performed by two cycling protocols for 30 minutes (intense interval and constant load) in young men increases Nrf2 protein expression (30). Conversely, Gomez et al stated that resistance training does not alter Nrf2 expression in young rats, whereas it decreased Nrf2 in older rats (31). A mouse study reported no changes in Nrf2 and glutathione peroxidase protein levels but decreased NQO1 protein expression in skeletal muscle after fatigued treadmill running (32). In one study, NrF2 protein concentration showed a significant decrease with acute AE in all regions of the brain and skeletal muscles. However, in another study, NrF2 protein concentration significantly increased in the hippocampus region of the brain in response to exercise training (33). The reasons for the difference in these results could be attributed to the type, duration, and intensity of sports activity. It seems that exercise with different intensities and duration can have different effects on Nrf2 expression.

In general, it was revealed that Nrf2 has a key role in the inhibition of NAFLD progression, and Nrf2 activators can potentially hinder and treat this disease (13). However, no studies reported the simultaneous effect of CR and AE on Nrf2 protein expression in patients with fatty liver, but the present study found that CR and AE can increase the expression of Nrf2 protein and reduce liver fat by reducing inflammation and liver damage, leading to the improvement of NAFLD.

Conclusion

The findings of this study showed that AE and CR simultaneously and separately reduce liver fat and simultaneously reduce liver inflammation and damage, which can be a reason for improving NAFLD. Likewise, it was observed that CR and AE alone could induce Nrf2 protein expression, but their simultaneous effect was more effective. According to the study that indicated Nrf2

improves NAFLD in various ways, it seems that the effect of AE in combination with CR can be a cost-effective and easy treatment for NAFLD.

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Author Contributions

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Investigation: Farzaneh Yadegari.
Methodology: Farzaneh Yadegari.
Project administration: Farzaneh Yadegari
Software: Farzaneh Yadegari.
Supervision: Farhad Rahmani Nia.
Writing – original draft: Farzaneh Yadegari and Farhad Rahmani Nia.
Writing – review & editing: Farzaneh Yadegari.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Approval

The study protocol was approved by the Research Ethics Committee of Sport Sciences Research Institute with code: IR.SSRI.REC.1400.1301.

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