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Concomitant Administration of L-carnitine and Performing High-Intensity Interval Training Effects on the Genes Involved in Mitochondrial Fusion and Apoptosis in Rat Liver

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Abstract

Mitochondria as dynamic organelles go through coordinated processes, including fusion and apoptosis. This study was designed to assess the effects of L-carnitine supplement and high-intensity training on the mitochondria fusion and apoptosis in liver tissue. Thirty-two Wistar rats were assigned into four groups, and eight rats were entered into each group, including the untreated control group (CTL), L-Carnitine group (LCAR; 200 mg/kg/day, i.p), High-intensity interval training group (HIIT), HIIT+L-Carnitine group (LCAR-HIIT; received 200 mg/kg/day L-Carnitine and performed HIIT). Real-Time PCR was used to quantify the expression of liver genes. LCAR-HIIT significantly decreased Parkin ($p<0.0001$), MFN-1 ($p=0.014$), Caspase-3 ($p=0.039$), Bax ($p<0.0001$) expression in the liver compared with the CTL. However, HIIT with LCAR significantly increased the expression of Bcl-2 compared with the CTL ($p=0.049$), while LCAR significantly reduced Bax expression compared to the CTL ($p=0.006$). LCAR-HIIT induced positive physiological changes in the liver through negative regulation of Bax and caspase-3. In addition, LCAR-HIIT may provide a new approach for ameliorating mitochondria fusion in the liver tissue.

Keywords: L-Carnitine, HIIT, Parkin, mitochondria fusion, Apoptosis

1. Introduction

Mitochondrial in eukaryotic cells acts as the ATP production site and also coordinates multiple metabolic reactions by the Krebs cycle and fatty acids metabolism. Mitochondria, as an essential organelle in the cells, regulate cell proliferation and survival, inflammatory pathways, and anti-inflammatory responses (Suliman, 2014).

Fission and fusion processes are related to the division or fusion of mitochondria. Still, the process of biogenesis of mitochondrial promotes mitochondrial mass and a regulatory network including Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), NRFs (Nuclear Respiratory Factors), mitochondria transcription factor A, MFN1, and MFN2 coordinate their dynamic (Litvinova et al., 2015, Palikaras et al., 2015, Norat et al., 2020). Mitochondrial biogenesis is thought to occur in response to increased workload, such as that present in training skeletal muscle or changes in the availability of substrates that occur during starvation (Suliman, 2014). Impaired quality control of mitochondria leads to the mitochondrial disturbance that contributes to some complications such as cardiovascular disease, diabetes mellitus, and aging. Parkin is an essential mediator of these processes (LaRocca et al., 2014).

Every cell in the body has a hidden program to destroy itself through apoptosis. Under normal circumstances, this program is kept off by inhibitory agents. One of the mechanisms of inhibition of apoptosis is the maintenance of molecules involved in apoptosis in organelles such as lysosomes and mitochondria (Vieira, 2003). Mitochondria are one of the main targets in apoptosis. Dynamic changes of proteins such as MFN2 and Drp1, and the apoptotic regulators Bax and Bcl-2 contributed to the mitochondrial fragmentation during apoptosis. Inhibition of the mitochondrial fission process is associated with activation of downstream caspases, thus delaying apoptosis, and overexpression of MFN1 and MFN2 also suppress the apoptotic process. The apoptotic proteins -caspase-3andBax- play a pivotal role in the prognosis of apoptosis in mitochondrial membrane permeability(Steiner et al., 2011, LaRocca et al., 2014, Palikaras et al., 2015, Dagda, 2018).Elevated levels of inflammatory cytokines, some growth factors, etc., reduced the expression of MFN2, while processes that increase energy consumption (such as exercise) increased its expression (Schrepfer and Scorrano,

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2016). Several other intracellular pathways, such as cell cycle progression, mitochondrial bioenergetics maintenance, apoptosis, and autophagy, have been shown modulated MFN2 (Filadi et al., 2018).

Carnitine (beta-hydroxy-gamma trimethyl ammonium butyrate) is known as a vitamin-like and amino-acid-like substance. It was documented that the L-isoform of carnitine is physiologically active. Its prominent role in the body is to promote the β-oxidation of lipids by transferring them to the mitochondrial matrix. Therefore, if carnitine is not present, most dietary lipids cannot be available for oxidation (Eskandari et al., 2004, Cha, 2008).

High-Intensity Interval Training (HIIT) is intense training period with short rest intervals that reduces the total training time and has aerobic-like effects (Nutrients Editorial, 2018).Steiner et al. showed that exercise increased mitochondrial biogenesis through PGC-1a and other mediators (Steiner et al., 2011). Carnevali et al. showed that HIIT increases mitochondrial fat transfer capacity (by increasing the activity of the enzyme carnitine palmitoyl transferase), which facilitates the beta-oxidation process (Carnevali, Eder et al., 2012). Also, HIIT improved the muscle oxidation direction of animals with hypertension (Holloway et al., 2015). Overall, HIIT increases energy efficiency and physical function (Kwak, 2013, Holloway et al., 2015, Fallahi et al., 2016). Kwak has described that exercise has a protective effect against apoptosis (Kwak, 2013).L-carnitine supplementation with regular aerobic exercise improved liver tissue apoptosis in type 2 diabetic subjects (Gholami et al., 2019).

Given the vital liver roles in the body's metabolic processes and the high content of mitochondria in the hepatocytes and the critical role of the liver in regulating metabolic processes and pathways related to exercise physiology, we evaluated the effect of L-carnitine supplementation and performing high-intensity exercise on the genes variations elaborated in mitochondrial dynamic and apoptosis in rat liver tissue to investigate how L-carnitine and intense intermittent exercise interact with each other.

2. Materials and Methods:

2.1. Materials

L-carnitine hydrochloride (Sigma, C0283-25G), EZ-10 Spin Column Animal Total RNA Miniprep extraction kit (BS82312, BIO BASIC), cDNA synthesis kit (Pars TousInc., A101161), RealtimeQPlus Master GreenMix (Ampliqon, 5000830high ROX™), primers were purchased from Metabion International Company.

2.2. Methods

Thirty-two Wistar rats (male, 8-9 weeks of age) were purchased from Physiology Research Center. Rats were kept in the standard condition with a temperature of 23 ± 2 °C and a light-dark cycle of 12/12 h. Rats had free access to food & water. The animals were monitored for one week to familiarize themselves with the new environment and separated into 4 groups (n = 8), including Untreated control group (CTL; with no intervention), the L-Carnitine group (LCAR; 200 mg/kg/day, i.p), High-Intensity Interval Training group (HIIT), HIIT+L-Carnitine group (LCAR-HIIT; received 200 mg/kg/day L-Carnitine and performed HIIT), randomly. The groups that needed to do the exercise were given a 2-week treadmill adaptation, and then the study was begun. The study lasted for four weeks. The current study was approved (Code number: IR.KMU.REC.1399.378) by the Ethics committee of Karman Medical University.

2.2.1. L-carnitine Administration:

L-carnitine is dissolved in the sterile normal saline and administrated by daily i.p injection (200 mg/kg) (Masoumi-Ardakani et al., 2020, Karabulut et al., 2021).

2.2.2. Training Protocol:

Familiarity of rats lasted for two weeks (15 meters/minute for 15 min). Exercise intensity was calculated by lactometer (Lactate Scout Company/Code: 37, Germany), which quantified the blood lactate levels directly after exercise, and levels > 6 mmol/L were considered high intensity (Verboven et al., 2019) (Table 1). For calculation of the intensity, the speed test starts with a warm-up of 10 meters per minute and then increases the speed (0.3 meter/minute) till exhaustion (Hu et al., 2021). Each session consisted ten 2 minutes work bouts/day at about 22 m/min, 29° slope separated by 2-minutes rest periods (5 days/week, 4-week) (Batacan et al., 2016). Finally, forty-eight hours after the final training session, the animals were anesthetized and sacrificed. The liver tissue of each rat was dissected, and rinsed with cold saline and frozen until future examinations.

Table 1. The blood levels of lactate measured directly after exercise

group	Lactate (mmol/L)
HIIT (n=8)	6.3
LCAR-HIIT (n=8)	6.1

2.2.3. Real Time-PCR carried out to measure genes expression

The Real-Time PCR method was carried out to determine the relative expression of target genes in this study. For this purpose, total liver RNA was extracted. The process of RNA extraction typically involves the destruction of cells in a chemical environment that simultaneously inactivates ribonucleases and then uses columns that can capture RNA molecules and pass other molecules through. In the final step, the RNAs were washed from the column using an elution buffer and collected in sterile tubes. Then the purity and concentration of the extracted RNA were set out by the Nano-drop device. The cDNA was synthesized from total RNAs, and to inhibit the RNase enzyme, RNasin (RNase inhibitor) was added to the reaction mixture. The obtained cDNA was then used for the next step. Real-time PCR was carried out with polymerase enzyme and specific primers (Table 2) for the target genes. The 18s rRNA gene was used as the housekeeping gene. After Real-time PCR, Ct values were obtained for samples and reference genes. The formula $2^{-\Delta\Delta CT}$was used to calculate relative expression of genes (Mohammadi et al., 2018).

Table 2. The sequences of primers to perform Real-time PCR

Gene	Forward	Reverse
Bax	ATCCAAGACCAGGGTGGCTG	CACAGTCCAAGGCAGTGGGA
Bcl-2	TATATGGCCCCAGCATGCGA	GGGCAGGTTTGTCGACCTCA
Caspase-3	GTGGAACTGACGATGATATGGC	CGCAAAGTGACTGGATGAACC
MFN1	ACCAATCCCGCTGGGGAGGA	TGGGGAGGTGCTGTCTCGGA
MFN2	TTCCACACCACTCCTCCGAC	AGCGTCCTCTCCCTCTGACA
NRF-1	TAGCCCATCTCGTACCATCAC	TTTGTTCCACCTCTCCATCAG
Parkin	CTGGCAGTCATTCTGGAC	CTCTCCACTCATCCGGTTT
18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

2.3. Statistical Analysis:

To analyze the data, we used SPSS version 22. We carried out the one-way analysis of variance (One-Way ANOVA) test, and to the pairwise comparison between groups, we performed Tukey's test. The data was expressed as Mean±SEM and the p value <0.05 was significant.

3. Results

The combination of LCAR+HIIT significantly reduced Parkin, MFN1, and caspase-3 expressions compared with the CTL (Figs 1, 3, and 5). The combination of LCAR+HIIT significantly reduced Parkin expression compared to the LCAR (p = 0.023) as well as HIIT (p = 0.012) groups (Fig 1). LCAR and combination of LCAR+HIIT significantly reduced Bax expression (Fig 6). The Bcl-2 expression in 3 intervention groups increased compared to the control, but only the combination of LCAR-HIIT caused a significant elevation (Fig 7). The expression of NRF-1 and MFN2 genes was not significant between the studied groups (Figs 2 and 4)

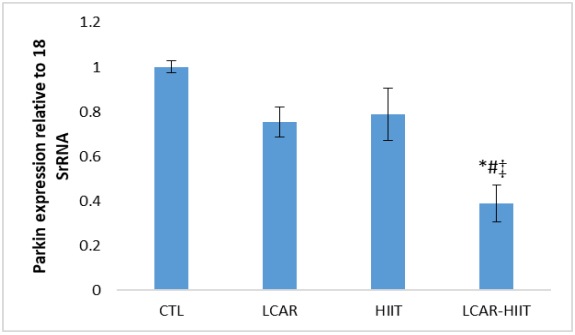

Figure 1. Parkin gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference. * Statistically significant compared to control, # statistically significant compared to LCAR, ‡ statistically significant compared to HIIT.Significant signs are the same in the rest of the figures.

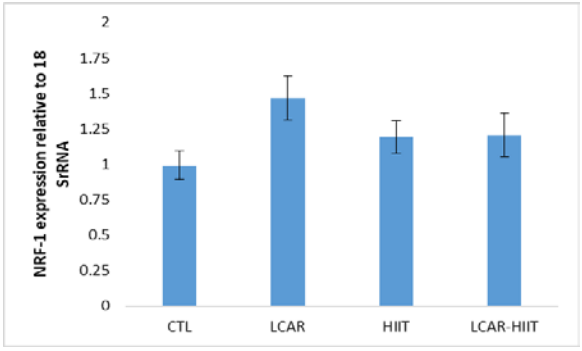

Figure 2. NRF-1 gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference.

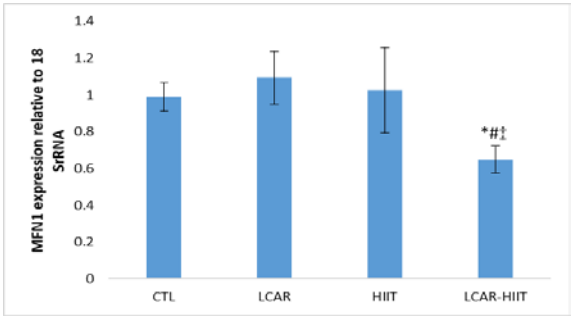

Figure 3. MFN1 gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference.

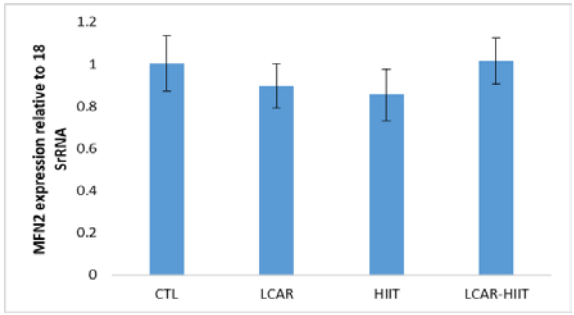

Figure 4. MFN2 gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference.

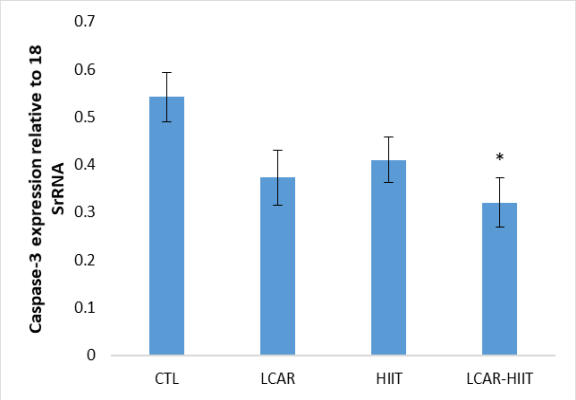

Figure 5. Caspase-3 gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference.

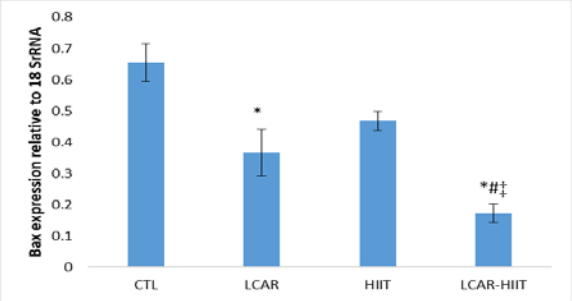

Figure 6. Bax gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference.

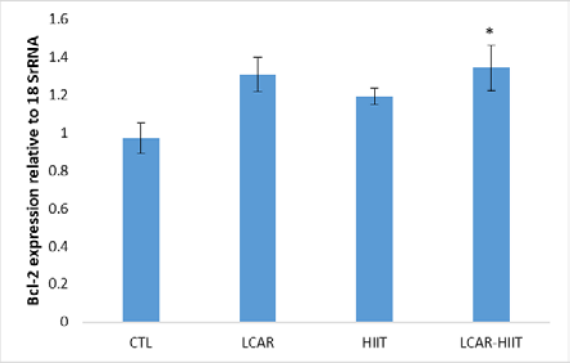

Figure 7. Bcl-2 gene relative expression (Mean±SEM) in 4 studied groups, including control (CTL), received L-carnitine (LCAR), high-intensity interval training (HIIT), and LCAR+HIIT. P< 0.05 was considered as significant difference.

4. Discussion

These current findings demonstrate the first evidence that L-CAR and HIIT contribute to the mRNA level of Parkin, MFN-1, Caspase-3, and Bax in liver tissue. Also, our current study provides evidence that HIIT and LCAR ameliorated Bcl-2 in liver tissue, possibly by suppressing the Parkin expression in the liver. Specifically, our results demonstrate that 1) HIIT and L-CAR decreased the mRNA expression level of apoptotic signaling, including Caspase-3, and Bax in the rat liver tissue, 2) L-CAR with HIIT elevated liverBcl-2expression, 3) L-carnitine and HIIT reduced the mRNA expression of MFN-1.

Based on the present study, four weeks of HIIT and L-CAR supplementation led to a remarkable reduction of Bax and the ratio of Bcl-2/Bax and also an increase in Bcl-2 levels of liver tissue. This indicates the protective effect of HIIT and LCAR supplementation through optimal regulatory pathways of apoptotic indices. Keleshian et al. reported that with increasing age, mRNA expression of pro-apoptotic Bax index and inflammatory and oxidative indexes in the frontal cortex of the brain increases, and Bcl-2 levels decrease (Keleshian et al., 2013). When there is no death receptor stimulus, the anti-apoptotic protein Bcl-2 is heterodimerized intracellularly with Bax. In case Bcl-2 overexpression, these molecules form homodimers and simultaneously inhibit apoptosis. Conversely, Bax overexpression leads to its homodimerization, resulting in increased sensitivity to apoptotic stimuli (Naim and Kaufmann, 2020). Therefore, disruption of the apoptotic balance and its shift to increased Bcl-2 following HIIT and L-CAR supplementation may be associated with the effects of support for liver tissue. In this regard, Um et al. showed that after 16 weeks of training, Bax levels in mice brains decreased, and Bcl-2 levels increased significantly (Um et al., 2008). Shirpour et al. observed that curcumin supplementation and HIIT showed an apoptosis inhibitory effect in hepatocytes (shirpour et al., 2017). Also, HumayunFard et al. reported that performing HIIT with selenium administration showed interactive properties on apoptosis proteins in rats (Humayun Fard et al., 2019). Although there are limited numbers of studies that evaluated the effect of HIIT and LCAR on the liver of rats and comparing the studies discussed with the results of our research, it appears that regular exercise has protective effects. Exercise training through phosphorylation of protein kinase B may reduce Bax and cytochrome C levels and therefore suppress apoptosis in the liver. Based on previous observations, HIIT was associated with a more significant impact on the development of liver function than continuous training (Rezaei et al., 2017). Jabbari et al. reported that L-carnitine supplementation and exercise training showed remarkable beneficial properties against apoptosis in diabetic rats (Jabbari et al., 2019). In sum, the results are not conclusive. Human elevated MFN1 during aging is reversed by running (Bori et al., 2012) or by six-week treadmill running in rats (Koltai et al. 2012); however, there were other human studies that demonstrated unaltered MFN1 in response to physical activity (Joseph et al., 2012, Gioscia-Ryan et al., 2016). Although they have shown that MFN1 expression up-regulated after cycling (12 weeks)(Konopka et al., 2014), after the same aerobic protocol, with the same duration and higher intensity, there was no increased MFN1 expression (Moreira et al., 2017).

Parkin, a cytosolic E3-ubiquitin ligase, functions in mitophagy. Specifically, the pol yubiquitination of Parkin substrates, such as MFN1/2, leads to their degradation by the proteasome. Induced mitochondrial fission and mitophagy after MFN1/2 destruction make a strong connection between mitochondrial dynamics to mitophagy. Mitophagy occurs in basal conditions constantly. Some specific physiological conditions can induce this process (Yoo and Jung, 2018).We demonstrated that Parkin is necessary for exercise-induced mitophagy. Interestingly, mitophagy induced after exercise was attenuated by training probably because of the promotion of basal

mitochondrial content and quality. It seems that Parkin is necessary for the preservation of basal mitochondrial function and increased good functioning mitochondria pool due to training adaptations (Chen et al., 2018). But HIIT and low-intensity interval training (LIIT), despite increasing PGC-1α and reducing DRP1, failed to improve mitochondrial fusion and fission (Ebadi and Damirchi, 2018).

One important issue that may be of interest in this study is the potential stress associated with performing high-intensity training and daily L-carnitine intraperitoneal injections. Previous studies have shown that exercise improves redox status by reducing pro-oxidant factors and increasing antioxidant intermediates. Poblete Aro et al. (2015) reported that HIIT (12 weeks) significantly reduced oxidative stress in diabetic patients (Poblete Aro et al., 2015). In a systematic review by Lu et al. (2021), they concluded that high-intensity exercise increased antioxidant capacity (Lu et al., 2021).

On the other hand, concerning L-carnitine administration, it was reported that L-carnitine intraperitoneal injections improved the redox state in diabetic rats (Masoumi-Ardakani et al., 2020). Hussein et al. (2013) reported that in high-fructose diet rats, L-carnitine increased liver catalase and superoxide dismutase (SOD) and reduced serum malondialdehyde level (Hussein et al., 2014). Also, L-carnitine administration reduced lipid peroxidation and increased serum SOD, catalase, and α-tocopherol in rats (Rajasekar, 2007). Lee et al. (Randomized clinical trial) linked reduced ROS levels to L-carnitine as its protective properties (Lee et al., 2014). It was also reported that L-carnitine i.p. administration promoted antioxidant defense status (Irat et al., 2003).

Finally, oxidative stress induces apoptosis and if HIIT and L-carnitine injection caused stress induction; therefore; we expect that apoptosis must increase in this group (LCAR-HIIT). On the contrary, we found that the expression of Caspase-3 and Bax reduced in LCAR-HIIT group, which indicates that there is probably no significant stress effect due to performing HIIT or L-carnitine injections.

In our study, in LCAR-HIIT group, both Parkin and MFN1 decreased in the same direction, so both mitophagy and mitochondrial fusion appear to be in equilibrium. By down regulation of Parkin we expected an increase in MFN1 levels, but the combination of exercise and carnitine administration reduced its expression, a balance has been struck between these processes. There are some limitations in this study; oxidative stress and antioxidants were not determined, and we did not examine the oxidative stress made by several injections and performing exercise.

5. Conclusion

In conclusion, the current study demonstrates that L-carnitine supplementation and performing HIIT improved positive physiological changes in the liver through negative regulation of Bax, caspase-3. In addition, LCAR-HIIT may provide a new approach for facilitating mitochondria fusion in the liver.

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