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Research Article

High intensity interval exercise alters muscle IL-18, FNDC5, and hepatic MMPs in animal model of steatosis: Evidence of skeletal muscle–liver crosstalk

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Abstract

Steatosis is a common disease worldwide. High intensity interval training (HIIT) may ameliorate steatosis, possibly through interactions between skeletal muscle and liver; however, mechanistic pathways are poorly understood. We aimed to determine potential mechanisms involved in skeletal muscle-liver crosstalk by measuring the gene expression of skeletal muscle interlukin-18 (IL-18) and fibronectin type III domain-containing protein 5 (FNDC5) and hepatic matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9). Thirty-two adult male Wistar rats were randomly divided into four group including normal control (C), high intensity interval training (HIIT), hepatic steatosis+ HIIT (HS+HIIT) and sedentary hepatic steatosis (SHS). HIIT was performed 5 days per week for 5 weeks. Tetracycline (140 mg/kg) was administered by gavage for 7 days to induce NAFLD. We found that HIIT and HS+HIIT increased skeletal muscle expression of FNDC5 relative to SHS group but the increase was attenuated in HS+HIIT. SHS increased muscle IL-18 expression relative to HIIT, HS+HIIT, and C. Expression of hepatic MMP-2 and MMP-9 increased significantly in SHS in comparison with C. There was a significant increase in MMP-9 in HIIT compared with C. Moreover, hepatic MMP-9 expression decreased in both HIIT and SHS+HIIT relative to SHS. MMP-2 decreased significantly in HIIT compared with SHS. Furthermore, muscle IL-18 gene expression was significantly associated with gene expression of hepatic MMP-2 and MMP-9. We conclude that HIIT-induced alteration of skeletal muscle-derived myokines may alter the gene expression of hepatic matrix metalloproteinases, collagenases involved in pathogenesis of liver diseases. Furthermore, steatosis may possibly influence myokine profiles in skeletal muscle. Accordingly, sk-eletal muscle-liver crosstalk is possibly targeted by HIIT and steatosis in terms of therapeutic approach.

Key Words: **High intensity interval training, Skeletal Muscle, Liver, Steatosis, Myokines, Matrix Metalloproteinases**

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a progressive disease (Rui, 2014) that is the most common chronic liver disorder worldwide and thus is a public health problem (Vernon, Baranova, Younossi, & Therapeutics, 2011). Triglyceride deposition in liver can destroy liver function and lead to liver complications including steatosis and steatohepatitis (Rui, 2014). Dysregulation of energy metabolism processes in the liver such as imbalance in the synthesis and elimination of hepatic triglyceride can contribute to the progression of NAFLD. Organ crosstalk may regulate the metabolic hemostasis of body in an endocrine, paracrine and/or autocrine manner (Delphan, Torabi , Delfan , & Delfan 2021; B. K. Pedersen, 2013). The largest organ of the body, skeletal muscle synthesizes and secretes myokines (e.g., proteins such as Irisin and IL-18) to establish a crosstalk with organs/tissues such as liver, adipose, bone and skin (B. K. Pedersen, 2013). In turn, the liver may communicate with skeletal muscle by its-derived cargoes (B. K. Pedersen, 2013; L. Pedersen et al., 2011).

MMP-2 and MMP-9, two members of matrix metalloproteinase family, increased in a steatosis model (Munsterman et al., 2018; Palladini et al., 2019). An increase in expression of pro-MMP-2 and pro-MMP-9 may mediate remodeling of hepatic matrix contributing to hepatocyte proliferation throughout liver regeneration after partial hepatectomy in rats (Kim, Mars, Stolz, & Michalopoulos, 2000). Furthermore, a human study showed that plasma levels of MMP-9 and the leukocyte expression of MMP-2 and MMP-9 increased in children with progressive NAFLD (Trojanek et al., 2020). Via their collagenase function,

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MMPs contribute to liver extracellular remodeling and degradation that is associated with liver pathology (Knittel et al., 2000). Also, a drug therapy study has shown that how modulating hepatic MMP-2 and MMP-9 via the down-regulation of NF-κB may reduce circulating cholesterol-induced hepatic inflammation by Naringenin supplementation, resulted in mitigating non-alcoholic steatohepatitis (NASH)-induced fibrosis (Chtourou et al., 2015).

Together with poor diet (excess caloric intake relative to caloric expenditure), sedentary lifestyle is a key risk factor for steatosis (Wattacheril & Sanyal, 2016). Skeletal muscle-derived myokines may manipulate the liver by altering the hepatic factors (B. K. Pedersen & Febbraio, 2012; B. K. Pedersen, 2013), and contribute to inhibit metabolic-related complications (Arias-Loste, Ranchal, Romero-Gómez, & Crespo, 2014). Skeletal muscle-derived FNDC5, the precursor of irisin, expressed in skeletal muscle (B. K. Pedersen & Febbraio, 2012; B. K. Pedersen, 2013), is an exercise-induced myokine, which has been emerged as holding therapeutic potential ameliorating NAFLD (Arias-Loste et al., 2014). It should be however noted that there is some skepticism over the direct contribution of skeletal muscle FNDC5 to human metabolic health (Timmons, Baar, Davidsen, & Atherton, 2012). Nevertheless, hepatocytes are a target of irisin (Lv et al., 2015; Park, Kim, Choi, Heo, & Park, 2015) and exercise yields beneficial effects on metabolic diseases such as NAFLD (Linden et al., 2016). Additionally, IL-18 has been suggested to activate AMP-activated protein kinase (AMPK) in skeletal muscle, resulting in elevating of fat oxidation (Lindegaard et al., 2013); AMPK has a wide range of influences including regulation of inflammation, insulin resistance and metabolic homeostasis (Lindegaard et al., 2013). Furthermore, IL-18 is involved in modulation of intramuscular lipid metabolism and hypertriglyceridemia in skeletal muscle (Lindegaard et al., 2018). An increase in plasma levels of IL-18 was observed after exercise in obese and non-obese women (Garneau et al., 2020). IL-18-induced MMP-9 expression is also implicated in the pathogenesis of diseases (Nold, Goede, Eberhardt, Pfeilschifter, & Mühl, 2003). What is more, the content and activity of hepatic MMP-2 and MMP-12 were attenuated in response to continuous aerobic exercise training, in association with attenuated liver fibrosis in rat with NAFLD-related fibrosis (Linden et al., 2016). These findings suggest that the liver is one of the major therapeutic targets of skeletal muscle in liver-related complication when it comes to organ crosstalk (Linden et al., 2016; Lv et al., 2015; Park et al., 2015). However, specific mechanisms responsible for the effect of HIIT on skeletal muscle-liver crosstalk and the mechanisms by which HIIT may improve NAFLD are poorly understood. Here, we aimed to examine the crosstalk between skeletal muscle and liver in response to five-week exercise training adaptation in rat with hepatic steatosis. To better understand the potential underlying mechanisms involved in muscle-liver interaction, we aimed to examine whether skeletal muscle signals to the liver and vice versa, and also to test the hypothesis that HIIT-induced alteration of muscle IL-18 and FNDC5-hepatic matrix metalloproteinase-2 and 9 are a potential underlying axis that attenuates hepatic steatosis progression in rats.

Materials and Methods

Animals

Thirty-two adult male Wistar rats (weighing 200–250 g) were purchased from Pasteur institute (Tehran, Iran). Animals were kept in standard conditions (22 ± 2°C, relative humidity 45-55%, and 12:12 h light-dark) and allowed free access to food and water ad libitum. All the procedures were conducted according to the Ethics Committee for the Use of Experimental Animals at the Baqiyatallah University of Medical Science. The rats were randomly divided into two groups: normal (n= 16) and steatosis (n= 16).

Induction of hepatic steatosis

The Steatosis group was divided into two groups: SHS and HS+HIIT (n=8 per group). Steatosis animals received Tetracycline by gavage for 7 days. Tetracycline dosage was 140 mg/kg dissolving in 2 ml of water. Fatty liver (steatosis) was confirmed by evaluating liver enzymes and H&E staining, and previously reported (Shabana, Ibrahim, Khadre, Elemam, & Zoology, 2012). Normal groups (C and HIIT groups, n=8) have received 2 ml of water as a control.

Exercise training protocol (HIIT)

The exercise training protocol was performed on a rodent treadmill for 5 consecutive weeks by HIIT and HS+HIIT groups. C and SHS groups did not perform any exercise activities (Kalaki-Jouybari et al., 2020). The rats in HIIT and HS+HIIT groups underwent a protocol with programmed time and progressive speed for 5 days per week, starting at the speed of 16m/min on the first day and reaching a speed of 40 m/min at the end day of the experiment. The protocol method was extracted from Kalaki-Jouybari et al. (2020). Every session started with a 5 min warm-up and finished with 5 min cool-down. The protocol is shown in Table 1.

Laboratory measurement

Animals were anesthetized and sacrificed by intraperitoneal injection of Ketamine (50 mg/kg) and Xylazine (10 mg/kg) 48 hours after the last training session and 10 to 12 hours of fasting. Gastrocnemius and liver tissue were removed. They were immersed in 4 volumes of RNAlaterTM fluid, then transferred to the laboratory. All samples were stored at −80 C until gene analysis.

Table 1. HIIT Training Protocol

Weeks of training	Intensity of warm up (m/min)	Number of intervals per session	Intensity of each interval (m/min)	Intensity of Recovery m/min)	Intensity of cool-down (m/min)
1	4	5	16-20	10	10
2	5	5	21-25	11	11
3	6	5	26-30	12	12
4	7	5	31-35	13	13
5	8	5	36-40	14	14

Histopathological examination of liver

To confirm HS in rats, one piece of liver tissue in the left lobe was fixed in 4% formaldehyde in phosphate-buffered solution and embedded in paraffin. Cross sections were cut to a thickness of 4–6 µm and stained with hematoxylin and eosin. Then, light microscopic examinations in a blinded fashion were performed by two pathologists and histological grades of NAFLD were determined (Brunt, Janney, Di Bisceglie, Neuschwander-Tetri, & Bacon, 1999).

Gene expression of IL-18, FNDC5, MMP-2 & MMP-9

mRNA quantitation was performed by quantitative real-time PCR. TRIZOL reagent (Ambion, Carlsbad, CA, USA) was used according to manufacturer instructions to isolate total RNA. DNA-free™ DNA Removal Kit (Invitrogen, Carlsbad, CA, USA) was used to remove DNA contaminants. To quantitate RNA, Biospec Nano Micro-volume UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan) was used.

To reverse transcribe RNA (100 ng) in duplicate, random hexamers and MultiScribe™ MuLV (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 µL were used as described by the manufacturer. The reverse transcription process of PCR was applied at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. cDNA duplication was subjected to qRT-PCR on a StepOne™ RT-PCR System (Applied Biosystems) using SYBR green qPCR master mix (Thermo Fisher Scientific). The specific primers corresponding to the selected mRNAs (IL-18, FNDC5, MMP-2, and MMP-9) are shown in Table 2. GAPDH was measured as the housekeeping gene and internal control. The following thermal profile was carried out for the qRT-PCR in three stages:

The first stage was applied at 95°C for 3 minutes (1 cycle); the second stage was performed at 95°C, 57°C, and 72°C for 30 seconds each (40 cycles); and the last stage was run at 95°C for 15 seconds and then 60°C for 1 hour (1 cycle). The $2^{-\Delta\Delta CT}$ method was used to determine the fold change for each gene after normalization to GAPDH (Livak & Schmittgen, 2001).

Statistical analysis

Data were analyzed using Graph Pad Prism version 8 (GraphPad Software, San Diego, USA) and statistical software SPSS version 21, and values were expressed as the mean ± standard error of mean. Kolmogorov-Smirnov test was used to analyze all the data for normality of distribution. Multiple comparisons were performed using one-way ANOVA followed by Tukey as post-hoc analysis. Correlation coefficients were calculated according to Pearson's correlation coefficient (r). Statistical significance level was set at $p < 0.05$.

Results

HIIT-induced FNDC-5 and suppressed IL-18 gene expression in gastrocnemius muscle

As shown in Figure 1, HIIT (P = 0.001) and HS+HIIT (P = 0.035) showed the significantly increase expression of FNDC-5 in the gastrocnemius muscle compared to the SHS group. Further, HIIT showed a greater enhancement of FNDC-5 compared to the HS+HIIT (p=0.001). According to Figure 2, the HIIT (p=0.001) and the HS+HIIT (p=0.001) groups showed significantly lower IL-18 expression in the gastrocnemius muscle compared to the SHS group. Moreover, in the SHS group, the expression of IL-18 was significantly elevated when compared to the C group (p= 0.001).

HIIT-induced hepatic alteration of MMP-2 & MMP-9

Figure 3 and Figure 4 show the expression of the hepatic MMP-2 and MMP-9 were markedly up-regulated (both p=0.001) in the

Table 2. Real-time PCR Primer Pequences

	Gene name	Primer sequence
1	MMP-2	Forward: GAACACCATCGAGACCATGC
		Reverse: GGTCCAGGTCAGGTGTGTAA
2	MMP-9	F: AGGATGGTCTACTGGCACAC
		R:GTGCAGGACAAATAGGAGCG
3	FNDC5	Forward: TGGCGAGATCCTGAACAACT
		Reverse: ACTCAAACAGCACCGTGAAC
4	IL-18	F: TGACAAAAGAAACCCGCCTG
		R: GGTCACAGCCAGTCCTCTTA
5	GAPDH	Forward: CAAGTTCAAGGGCACAGTCA
		Reverse: CCCCATTTGATGTTAGCGGG

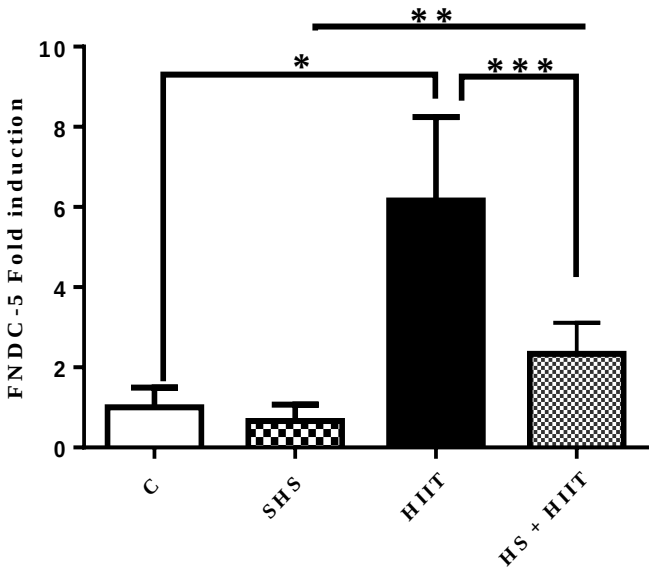

Figure 1. FNDC5 mRNA Expression in the Gastrocnemius Muscle of Treatment Group. Data Are Presented as means ± SED. P ≤ 0.05. * p < 0.05 Compared to Control (C) Group, ** p < 0.05 Compared to SHS (Sedentary Hepatic Steatosis) Group, and *** p < 0.05 Compared to High Intensity Interval Training (HIIT) Group. (LPRT and UPRT)

SHS group in comparison with the C group (Figures 3 and 4). Also, the expression of MMP-9 was significantly higher in the HIIT group than in that in the C group (p=0.001). The HIIT (p=0.001) and the HS+HIIT (p=0.001) displayed a significant decrease in mRNA expressions of MMP-9 in comparison with the SHS group. Moreover, MMP-2 was significantly lower in HIIT compared to the SHS group (p=0.009).

Association of muscle IL-18 with hepatic MMP-2 and MMP-9

Figure 5 shows that there is a significant positive correlation between expression of muscle IL-18 and hepatic MMP-2 (r = 0.578, P = 0.001). Likewise, Figure 6 shows the significantly positive association between muscle IL-18 and hepatic MMP-9 (r = 0.793; P = 0.001).

Association of muscle FNDC5 with hepatic MMP-2 and MMP-9

As shown in Figure 7, there was no correlation between muscle FNDC5 and hepatic MMP-2 (r = -0.214; P = 0.239). There was no correlation between muscle FNDC5 and hepatic MMP-9 (r = -0.051; P = 0.782; Figure 8).

Discussion

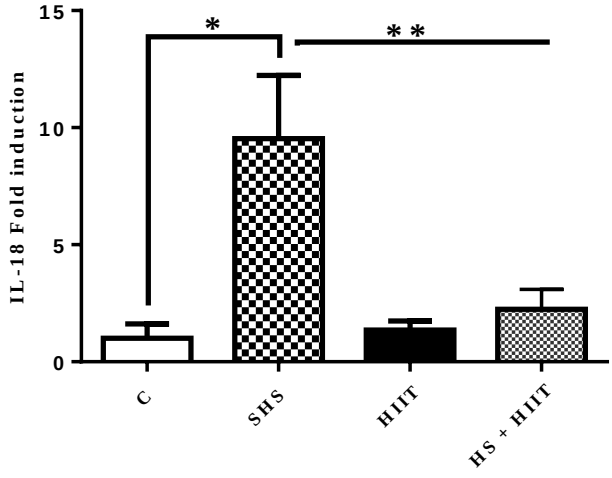

Figure 2. IL-18 mRNA Expression in the Gastrocnemius Muscle of Treatment Group. Data are Presented as Means ± SED. P ≤ 0.05. * p < 0.05 Compared to Control (C) Group, and ** p < 0.05 Compared to SHS (Sedentary Hepatic Steatosis) Group.

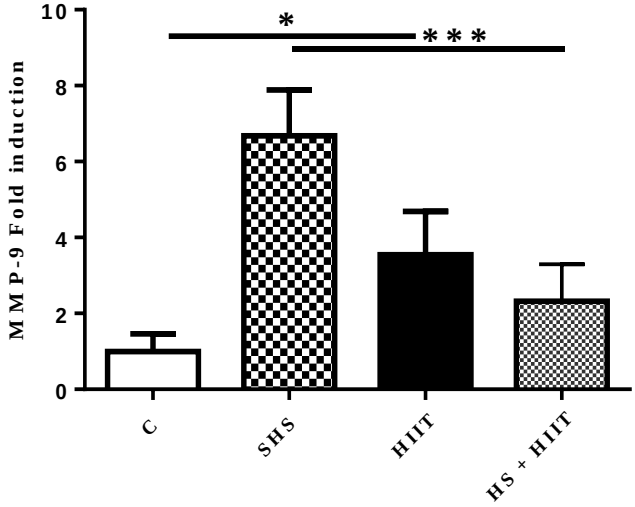

Figure 3. MMP-9 mRNA Expression in the Gastrocnemius Muscle of Treatment Group. Data Are Presented as Means ± SED. P ≤ 0.05. * p < 0.05 Compared to Control (C) Group, and *** p < 0.05 Compared to High Intensity Interval Training (HIIT) Group.

Our findings contribute to the understanding of how the skeletal muscle and liver communicate in response to HIIT and hepatic steatosis in an animal model. The major findings of the present study are that HIIT decreases skeletal muscle expression of IL-18, increases skeletal muscle FNDC5 expression, and decreases both hepatic expression of MMP-2 and MMP-9 relative to SHS. SHS upregulates skeletal muscle expression of IL-18, decreases skeletal muscle FNDC5 and increases hepatic expression of MMP-2 and MMP-9. Additionally, we show that muscle IL-18 but not FNDC5 is correlated with liver MMP-2 and MMP-9. This finding may represent the evidence regarding skeletal muscle-liver crosstalk to confirm how HIIT-induced alteration mRNA expr-

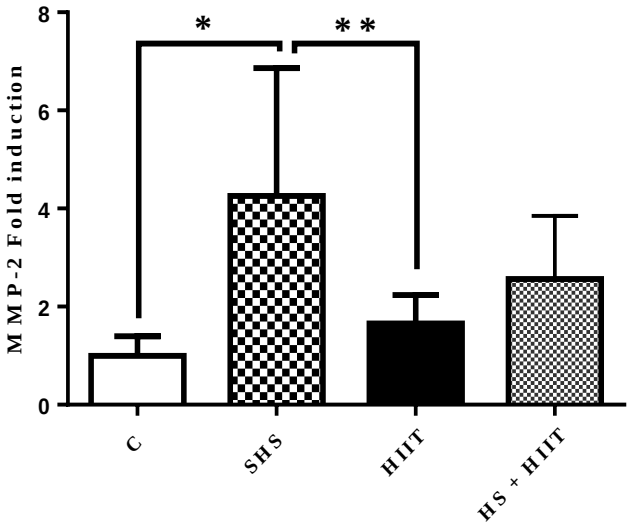

Figure 4. MMP-2 mRNA Expression in the Gastrocnemius Muscle of Treatment Group. Data Are Presented as Means ± SED. P ≤ 0.05. * p < 0.05 Compared to Control (C) Group, and ** p < 0.05 Compared to SHS (Sedentary Hepatic Steatosis) Group.

-ession of myokines including FNDC5 and IL-18 may possibly manipulate the profile of markers in the liver, as a distant organ, including MMP-2 and MMP-9, and how SHS-induced changing in extracellular matrix remodeling-related markers including MMP-2 and MMP-9 may possibly govern skeletal muscle-derived factors (FNDC5 and IL-18) in sedentary rats with hepatic steatosis.

Exercise-induced skeletal muscle contraction-derived cargos are likely involved in mediating of the beneficial impact of exercise in attenuating NAFLD development (Arias-Loste et al., 2014). It was reported that the crosstalk between skeletal muscle and liver is remarkably modulated by exercise (B. K. Pedersen & Febbraio, 2012; B. K. Pedersen, 2013). There is evidence that FNDC5 (Moreno-Navarrete et al., 2013), and IL-18 (Plomgaard, Penkowa, & Pedersen, 2005) are expressed in skeletal muscle, and are translated into signal peptides in response to skeletal muscle contraction to establish local and distant interaction with other organs (B. K. Pedersen & Febbraio, 2012; B. K. Pedersen, 2013). This evidence underpinned our rationale to investigate putative regulators of crosstalk: skeletal muscle-derived IL-18, FNDC5 and hepatic factors of MMP-2, MMP-9, in rats with hepatic steatosis, as well as the influence of HIIT.

IL-18 mediates a cascade of pro-inflammatory signaling (Novick, Kim, Kaplanski, & Dinarello, 2013). Lack of IL-18, and its receptor in the liver, results in hepatic fat deposition in the liver of mice fed with chow, suggesting that IL-18 is involved in hepatic homeostasis in a healthy diet setting (Lana et al., 2016). Evidence showed an increase in body weight gain and inflammation development in skeletal muscle and liver of mice with genetic deletion of IL-18 (Lindegaard et al., 2013; Netea et al., 2006). NAFLD and steatohepatitis are accompanied by dislip-dyslipidemia in IL-18 knockout mice (Yamanishi et al., 2016). Furthermore, dyslipidemia improved by recombinant IL-18 administration resulting in inhibiting the steatohepatitis progression in these mice (Yamanishi et al., 2016). Our findings showed that exercise decreased IL-18 expression in skeletal muscle of HIIT and HS+HIIT groups, suggesting that HIIT may modulate inflammation development in skeletal muscle. Interestingly, we found IL-18 expression remarkably increased in sedentary steatosis group, suggesting that liver dysfunction may alter the profile of distant organ cargos such as skeletal muscle-derived factors.

Previous work has shown that liver fat accumulation was attenuated by irisin in mice fed with high fat diet (Xiong et al., 2015). Evidence suggests that FNDC5 may mediate hepatic glucose (Liu et al., 2015; Mo et al., 2016) and lipid metabolism (Mo et al., 2016) by decreasing lipogenesis and gluconeogenesis in the hepatic cells via activation of PI3K/Akt (Liu et al., 2015) and AMPK (Mo et al., 2016), resulting in metabolic homeostasis improvement. Low systemic levels of irisin was also associated with NAFLD (Shanaki et al., 2017). It has also been shown that

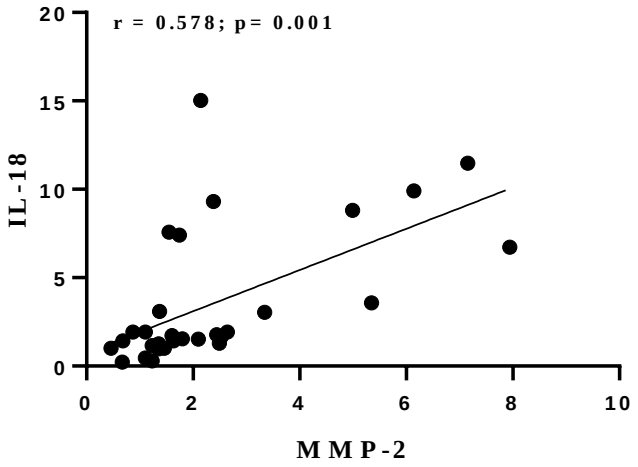

Figure 5. Association of IL-18 and MMP-2. Pearson's correlation coefficients of Gastrocnemius IL-18 with hepatic MMP-2.

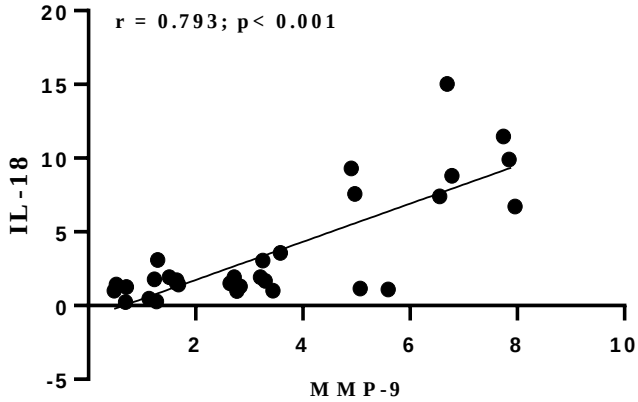

Figure 6. Association of IL-18 and MMP-9. Pearson's correlation coefficients of Gastrocnemius IL-18 with hepatic MMP-9.

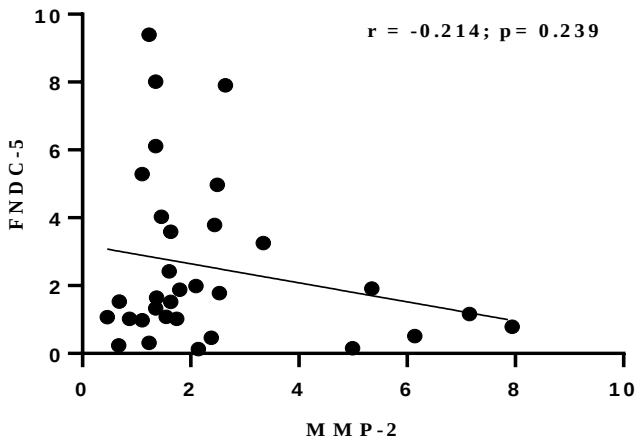

Figure 7. Association of FNDC5 and MMP-2. Pearson's correlation coefficients of Gastrocnemius FNDC5 with hepatic MMP-2.

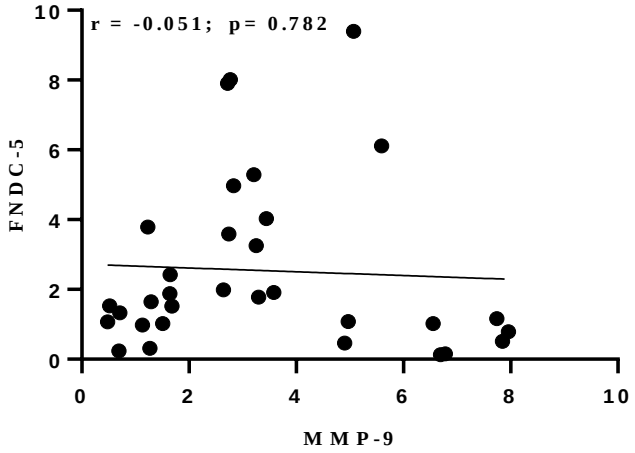

Figure 8. Association of FNDC5 and MMP-9. Pearson's correlation coefficients of Gastrocnemius FNDC5 with hepatic MMP-9

HIIT increases systemic irisin in adult male Wistar rats (Shirvani, Arabzadeh, 2020; Shirvani, Rahmati-Ahmadabad, 2019). Here, we show an increase in irisin expression in HIIT group compared to C, HS+ HIIT and SHS groups. We also found that SH+HIIT group exhibited a significant increase in skeletal muscle irisin expression compared to the SHS group. These findings suggest that HIIT may modulate the profile of skeletal muscle-derived irisin occurring in hepatic steatosis.

High MMP-9 mRNA expression in peripheral blood leukocytes and its higher plasma levels are found in children with NAFLD (Trojanek et al., 2020). MMP-9 is also highly upregulated (protein and mRNA expression) in injured liver tissue (Abe et al., 2008). Likewise, upregulation of systemic MMP-9 and the leukocyte expression of MMP-2 and MMP-9 were observed in progressive NAFLD (Trojanek et al., 2020) and contributed to pathological conditions of liver. Furthermore, it was demonstrated that exercise-induced suppression of hepatic MMP-2 and MMP-12 contribute to lower liver fibrosis progression in rats with NAFLD-related fibrosis (Linden et al., 2016). This evidence is in agreement with our findings. Here, we showed hepatic steatosis significantly upregulated hepatic MMP-2 and MMP-9 compared to C group. We also showed expression of MMP-2 decreased in HIIT group and tended to be lower in HS+HIIT group. However, a remarkable decrease in hepatic MMP-9 expression was observed in both HIIT and HS+HIIT groups. Accordingly, HIIT may govern MPPs profiles in liver to mitigate hepatic steatosis progression.

It has been previously shown that IL-18, a mediator of MMP-9, increased production of MMP-9 by human peripheral blood mononuclear cells and induced its secretion from whole blood cultures of human, implicating a role for IL-18 and MMP-9 in the pathogenesis of diseases (Nold et al., 2003). In addition, serum concentrations of IL-18 are associated positively with serum con-centrations of MMP-2 in non-alcoholic steatohepatitis patient, suggesting that IL-18 may contribute to liver fibrosis via MMP-2 upregulation (Olusi, Abdeen, George, & Research, 2012). We also observed a strong positive correlation of muscle IL-18 with hepatic MMP-2 and MMP-9, suggesting communication between skeletal muscle and liver in an endocrine manner, a process that may inhibit the progression of hepatic steatosis with exercise training.

Conclusion

In summary, this study suggests that the liver is an important target of exercise – and possibly skeletal muscle myokines –in NAFLD. While further research is required, exercise may sensitize FNDC5 actions and desensitize IL-18 actions in skeletal muscle, which in turn may signals to coordinate organs crosstalk for improving hepatic steatosis disease. Alteration of muscular myokines may initiate a dialog with liver and thereby inhibiting MMP-2 and MMP-9 mRNA expression to mitigate hepatic steatosis progression; this may indicate skeletal muscle and liver crosstalk as therapeutic target for NAFLD. Whereas our findings support the existence of a muscle-liver interaction, a challenge exists to understand how skeletal muscle-derived myokines respond to exercise, 'talk' to the liver and mitigate or reverse steatosis. These findings added to body literature that skeletal muscle-derived IL-18 and FNDC5 might be involved in improvement of hepatic steatosis, possibly via altering the profile of MMP-2 and MMP-9 in the case of IL-18, after 5 week HIIT. Hence, HIIT-induced myokines may be counted as nexus to contribute to a novel therapeutic option for nonalcoholic fatty liver disease setting. Additionally, hepatic steatosis could influence muscle inflammation profile (e.g., FNDC5 and IL-18). Further research is required to understand the potential role of FNDC5 and IL-18 and other myokines in altering liver factors and vice versa in a crosstalk manner.

What is already known on this subject?

Exercise training including HIIT improves NAFLD. However, potential HIIT-induced mechanisms, including skeletal muscle-liver crosstalk, are poorly understood.

What this study adds?

HIIT may induce skeletal muscle to signal to the liver, improving hepatic steatosis. The outcomes of the present study not only add to body literature of organ-organ crosstalk in chronic disease prevention and treatment, but also help to understand how HIIT-induced signals (myokines) impact the liver, and ultimately mitigate steatosis progression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experimental protocols were approved by the Ethics Committee of the Baqiyatallah University of Medical Science.

Informed consent All authors consent to this manuscript submission.

Author contributions

Conceptualization: M.D1., N.D.; Methodology: D.W., F.R.; Software: D.W., M.D1.; Validation: N.D.; Formal analysis: M.D2.; Investigation: F.M., A.N.; Resources: M.D1.; Data curation: A.N.; Writing - original draft: M.D2., D.W.; Writing - review & editing: M.D1.; Visualization: J.A., AH.P.; Supervision: F.R.; Project administration: A.N.; Funding acquisition: F.R.

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