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Lactate/GPR81 signaling is associated with high intensity interval training-induced modulation of hepatic fatty acid oxidation and ferroptosis-related markers in type 2 diabetic rats

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ABSTRACT

Objective(s): Type 2 Diabetes (T2D) can lead to hepatic injury through mechanisms involving impaired β-oxidation and ferroptosis. This study investigated whether high-intensity interval training (HIIT) could improve T2D-induced hepatic injury by enhancing hepatic lactate/GPR81 signaling and MCT-1 expression, thereby reducing ferroptosis-related factors in male rats.

Materials and Methods: Twenty-four male Wistar rats were randomly assigned to four groups: T2D, HIIT, T2D+HIIT, and control (CTL). T2D was induced with low-dose streptozotocin (30 mg/kg) and a 2-month high-fat diet. HIIT was performed at 80–100% of maximum running speed for eight weeks, five sessions per week. Hepatic expression of GPR81, MCT1, PPARα, HNF4α, CPT1A, EHHADH, and ACSL4, hepatic lactate levels, anti-oxidant enzyme activity, serum metabolic indicators, and HOMA-IR were also assessed.

Results: T2D significantly reduced hepatic GPR81, MCT1, PPARα, HNF4α, CPT1A, EHHADH, and anti-oxidant enzyme activities, while increasing fasting blood glucose, triglycerides, HOMA-IR, serum lactate, and hepatic ACSL4 expression. HIIT reversed these changes, decreasing ACSL4 expression and increasing hepatic lactate and the expression of proteins involved in lactate transport and β-oxidation.

Conclusion: In diabetic rats, HIIT also reduced insulin resistance and enhanced antioxidant defense mechanisms. HIIT ameliorates hepatic injury induced by T2D, associated with modulation of fatty acid oxidation-related markers, reduced ferroptosis-related factors, and enhanced hepatic lactate/GPR81 signaling.

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Introduction

The global obesity epidemic has led to a rise in metabolic disorders, posing a significant public health concern and acting as a primary catalyst for diseases such as type 2 diabetes (T2D) (1). T2D is considered the most common metabolic disorder and is expected to be the seventh leading cause of death worldwide by 2030 (2). In addition to other problems, hepatic injury is common in obese patients with T2D (3).

A primary underlying cause of hepatic injury in patients with T2D is excessive fatty acid accumulation in the liver, which may be linked to increased hepatic triglyceride (TG) synthesis and decreased beta-oxidation (4). Insulin resistance is a key factor contributing to reduced hepatic beta-oxidation, potentially leading to rapid progression of hepatic injury to hepatocellular carcinoma (HCC) (5).

The peroxisome proliferator-activated receptor alpha (PPARα) plays a crucial role in fatty acid metabolism (6). Studies have shown that its agonist can lower blood glucose levels in mice fed a high-fat diet (HFD) (6). Beta-oxidation-related genes, such as hepatocyte nuclear factor 4α (HNF4α), have been identified as potential PPARα targets (7). HNF4α, an essential regulator of metabolic homeostasis, transcribes genes involved in lipid metabolism (8). A mutation in the gene encoding this factor has been reported to reduce beta-oxidation (8). HNF4α may play a role in the initiation and progression of hepatic injury. It could be a promising treatment target (9), as animals lacking HNF4α have been shown to accumulate fat in the liver (10). Increased HNF4α expression prevents HFD-induced hepatic steatosis (11). Furthermore, in HFD-fed diabetic NASH models, its

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hepatic expression is significantly reduced (11). Carnitine palmitoyltransferase 1A (CPT1A) is a primary marker of beta-oxidation activity (12, 13), and HNF4α has been shown to increase CPT1A expression (14). In addition, PPARα induces not only HNF4α but also the enzyme EHHADH (15). This enzyme is part of the classical peroxisomal fatty acid oxidation pathway, coordinating mitochondrial and peroxisomal oxidative pathways (15), and is involved in hepatic beta-oxidation.

Recently, reduced beta-oxidation has been shown to exacerbate hepatic injury by activating hepatic ferroptosis (16). Ferroptosis is a non-apoptotic programmed cell death characterized by the accumulation of reactive oxygen species in lipids, ultimately leading to membrane damage (17). Ferroptosis can induce or exacerbate hepatic injury through increased lipid peroxidation (17), inflammation (18), and oxidative stress (18). Acyl-CoA synthetase long-chain family member 4 (ACSL4), a member of the long-chain acyl-CoA synthetase family, is essential for ferroptosis (19, 20). HFD induces ferroptosis and impairs cardiac function by increasing ACSL4 expression (21). ACSL4 reduces beta-oxidation by inhibiting PPARα (22, 23).

G protein-coupled receptor 81 (GPR81) is an endogenous receptor for lactate that signals with PPARs (24) and CPT1 (25). This receptor plays a crucial role in adipose tissue lipolysis (26) and is significantly increased in the liver of fasting mice, but markedly reduced in obese mice (27). GPR81 improves hepatic injury by increasing hepatic CPT1 expression (27). It also enhances MCT1 expression (28), and lactate entering the liver through MCT1 inhibits ACSL and reduces ferroptosis (22). Overall, GPR81 is crucial for hepatic lipid homeostasis, and its activation in the liver may be a promising strategy for treating obesity and associated metabolic disorders (29).

One way to increase GPR81 is through physical exercise (30). Research has shown that high-intensity interval training (HIIT) can increase GPR81 expression (31), causing blood lactate levels to rise from a baseline of 3 mM to 15 mM in mice, the highest among all exercise training methods (31). Additionally, hepatic MCT1 expression is significantly increased during this type of exercise (32).

Given the significant role of ferroptosis in hepatic injury and the fact that decreased beta-oxidation is a primary cause of this disorder, we hypothesized that HIIT, by enhancing lactate-dependent beta-oxidation, could inhibit ferroptosis and reduce hepatic injury. In this study, we aimed to investigate whether HIIT, mediated by lactate and improved beta-oxidation signaling through increased PPARα expression and decreased ACSL4 expression, could affect ferroptosis-related factors and ameliorate hepatic injury. To this end, we studied the effects of 8 weeks of HIIT on the hepatic expression of lactate induced beta oxidation markers (i.e. GPR81, MCT1, HNF4α, PPARα, CPT1, EHHADH) and the main ferroptosis marker (ACSL4), as well as serum concentration of biochemical markers (such as lactate, triglycerides (TG), glucose, and insulin) and the activity of anti-oxidant enzymes (i.e. superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT)) in male Wistar rats with T2D.

Materials and Methods

Animals

This interventional-experimental investigation involved 24 male Wistar rats, each weighing 200-250 grams, sourced from the animal facility of Kerman University of Medical Sciences. Animals were housed under controlled environmental conditions, with temperature maintained at 22±2 °C, relative humidity at 50–60%, and a 12-hr light/12-hr dark cycle. Animals were kept in standard cages with appropriate bedding. Food and water were provided ad libitum throughout the study. Animals were monitored daily to ensure health and welfare. Animals were randomly allocated into four groups (six per group): control (CTL), T2D, HIIT, and T2D + HIIT. Randomization was performed before the start of the experiment using a computer-generated randomization schedule (e.g., a random number generator), assigning animals to experimental groups at random to minimize selection bias. The Ethics Committee of Kerman University of Medical Sciences approved the entire procedure (Ethics code: IR.KMU.AEC.1403.017). All animal experiments were conducted in accordance with institutional guidelines and regulations and were reported in compliance with the ARRIVE guidelines.

Diabetes induction

Animals in the T2D and T2D+HIIT cohorts received a high-fat diet (HFD) containing 60% fat, 20% carbohydrates, and 20% protein for two months. They then received a 30 mg/kg streptozotocin (STZ) injection. STZ was freshly dissolved in cold 0.1 M citrate buffer (pH 4.5) immediately before use and administered at 1 mL/kg. Animals were fasted overnight (12 hr) with free access to water before STZ administration and remained fasted until 12 hr after injection. Fasting blood glucose (FBG) was measured from tail vein blood with a glucometer three days after STZ injection, following a 12-hr overnight fast. Animals with FBG levels greater than 300 mg/dL were classified as diabetic.

Training protocol

During the familiarization phase, the HIIT+ T2D and HIIT groups used a treadmill daily for 5 days, exercising for 10 min each day at a zero incline and a pace of 8 meters per minute. An incremental running test was conducted to determine the rats' maximal speed (Vmax). In this experiment, the rats ran for 2 min at 6 meters per minute, increasing the speed by 2 meters per minute every subsequent 2 min until they could no longer maintain the pace. The final attempt of the rats was recorded as Vmax. The main training regimen lasted eight weeks, five days per week. At the beginning and end of each session, the rats ran on a treadmill for 5 min at 40–50% of Vmax to warm up and cool down. The rats' Vmax was reevaluated every two weeks to guide the design of the next training session. The total training duration started at 12 min in the first week and increased to 30 min in the final week. Also, we did not use electrical stimulation. Table 1 presents the training program details (33-41).

Sampling

Rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After confirming deep anesthesia by the absence of the pedal withdrawal reflex, animals were sacrificed by rapid decapitation. The liver tissue was carefully excised, cryopreserved in liquid nitrogen, and stored at -80 °C for further study. Serum lactate (Abcam, UK Cat# ab65331) and insulin (Crystal Chem Inc., USA; Cat# 90060)

Table 1. High-intensity interval training (HIIT) protocol: Weekly progression of the laod

Week	Intervals	HIgh-intensity interval duration (min)	Low-intensity interval duration (min)	HIgh-intensity interval velocity (%Vmax)	Low-intensity interval velocity (%Vmax)	Total exercise time within a session (min)	Weekly load (AU)
1	4	2	1	80	50	12	42
2	4	2	1	85	50	12	44
3	6	2	1	85	50	18	66
4	6	2	1	90	50	18	69
5	8	2	1	90	50	24	92
6	8	2	1	95	50	24	96
7	10	2	1	95	50	30	120
8	10	2	1	100	50	30	125

concentrations, and hepatic lactate concentration (Abcam, UK Cat# ab65331), were measured using enzyme-linked immunosorbent assay (ELISA) following the manufacturer's guidelines.

An enzymatic colorimetric assay was used to quantify serum TG levels (Abcam, UK Cat# ab65336).

The homeostasis model assessment (HOMA) was used to evaluate insulin resistance (HOMA-IR). The HOMA-IR score was calculated using the formula: HOMA-IR= [(fasting glucose (mmol/l) × fasting insulin (μU/ml))/22.5]. Fasting blood glucose (FBG) was measured using a glucometer (AccuChek, USA).

Liver SOD activity was determined using the method of Paoletti and Mocali (42). This method involves superoxide radicals produced during hypoxanthine oxidation by xanthine oxidase reacting with 2-(4-iodophenyl)-3-(4-nitrophenol)- 5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. SOD activity was measured by inhibition of this reaction. One unit of SOD is the amount of enzyme needed to dismutate 50% of the superoxide radicals.

GPx activity was evaluated using the method described by Paglia and Valentine (43). GPx catalyzes the oxidation of glutathione, which is then reduced in the presence of glutathione reductase and NADPH. The extinction of NADPH was read at 412nm to indicate glutathione peroxidase activity.

Western blot

We evaluated the expression of GPR81 (Abcam, UK Cat#ab106942), MCT1 (Abcam, UK Cat# ab324262), CPT1A (Abcam, UK Cat# ab128568), ACSL4 (Abcam, UK Cat# ab155282), HFN4α (Abcam, UK Cat# ab201460), PPARα (Abcam, UK Cat# ab41928), and EHHADH (Abcam, UK Cat# ab123490) through western blot analysis. Liver tissue samples were homogenized using a lysis buffer. The whole protein extract was then centrifuged at 15,000 rpm for 5 min. The protein concentration in the resulting supernatants was measured using Bradford's technique. Sixty micrograms of protein from the lysates were loaded onto a 12% SDS-PAGE gel and then transferred to a PVDF membrane (Chemicon Millipore Co., Temecula, USA). The membranes were blocked with a 2% blocking reagent from the enhanced chemiluminescence (ECL) Advanced Kit (Amersham Bioscience Co., Piscataway, USA) and then incubated with primary antibodies overnight. After three washes with TTBS (Tris-buffered saline with Tween 20), the blots were incubated with a secondary antibody, specifically rabbit IgG conjugated to horseradish peroxidase (HRP) at a dilution of 1/3000 (Cell Signaling Technology Co., New York, USA), for one hour at room temperature. The reactive bands were detected using a chemiluminescence kit reagent (Amersham Bioscience Co., Piscataway, USA). Blot quantification was performed in ImageJ. Rabbit anti-beta-actin (dilution 1:1000, Cell Signaling Technology Co., New York, USA) served as the internal control.

Statistical analysis

The sample size (n=6 per group) was determined based on previous studies (44, 45) using similar high-fat diet/STZ-induced T2D models with exercise interventions, where comparable group sizes were sufficient to detect significant molecular and metabolic differences. The results of this investigation are presented as mean±SEM. We used the Shapiro–Wilk and Levene's tests to assess normality and homogeneity of variance, respectively. We used repeated-measures ANOVA to assess changes in FBG and body weight. In contrast, one-way ANOVA was used for the remaining parameters, with the Brown–Forsythe test when data were heteroscedastic. *Post hoc* pairwise comparisons were performed using Tukey's test for homogeneous data and Games-Howell for heterogeneous data, with the significance level set at 0.05.

Results

Serum FBG and insulin, body weight, and HOMA-IR

Repeated-measures ANOVA (group*time interaction) revealed significant differences in FBG ($F_{3,20}$=85.21, P<0.001) and body weight ($F_{3,20}$=96.32, P<0.001) among the experimental groups. Our findings showed that FBG and body weight increased after diabetes induction compared with baseline in both the T2D and T2D + HIIT groups, compared with the CTL group (P<0.001). Furthermore, at month 4, the T2D+HIIT group had lower FBG (P<0.001) and higher body weight (P<0.05) than the T2D group. In addition, at month 2, after T2D induction and before the training intervention, FBG and body weight did not differ significantly between the T2D and T2D+HIIT groups (P > 0.05), indicating comparable diabetic severity between the two diabetic groups before training (Figure 1A&B).

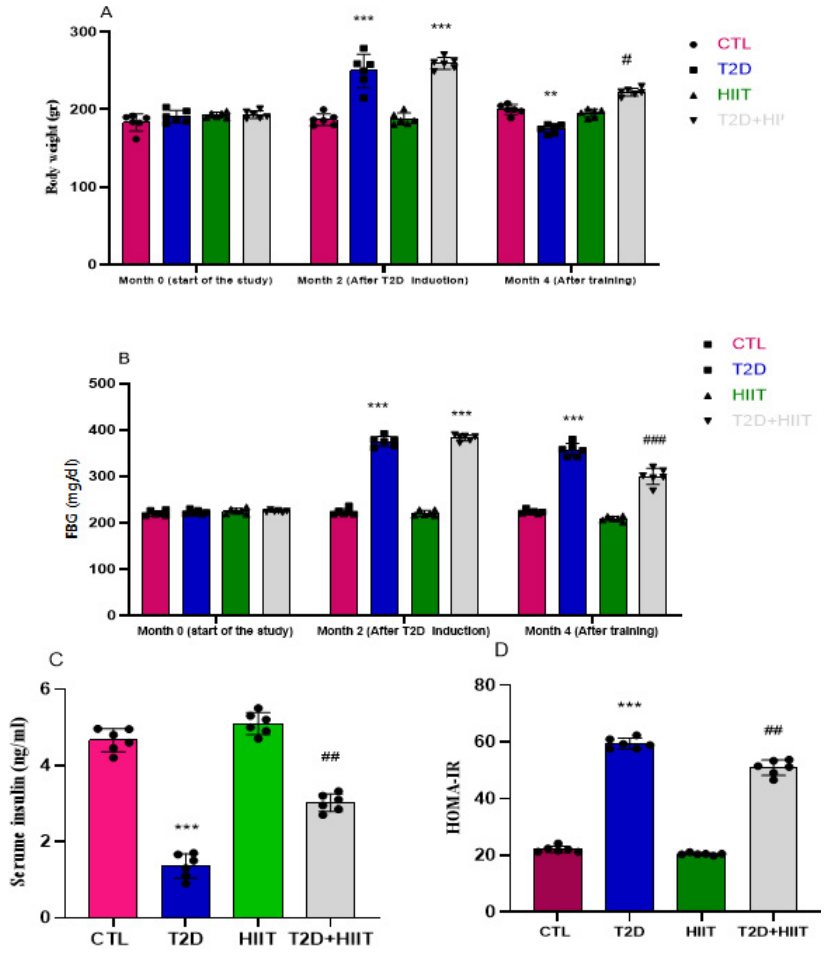


Figure 1. Male wistar rats body weight (A), fasting blood glucose (FBG) levels (B), serum insulin levels (C), and homeostatic model assessment of insulin resistance (HOMA-IR) (D). Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean ± standard error of the mean (n=6), with p<0.05 considered statistically significant
CTL: Control, T2D: Type 2 Diabetes, HIIT: High-intensity interval training.*** *P*<0.001 vs CTL.# *P*<0.05 , ## *P*<0.01 and ### *P*<0.001 vs T2D

Additionally, blood insulin levels ($F_{3,20}$=35.36, *P*<0.001) and the HOMA-IR index ($F_{3,20}$=96.1, *P*<0.001) differed significantly between groups. Insulin levels in the T2D group were significantly lower than in the CTL group (*P*<0.001). Conversely, the T2D+HIIT group showed higher insulin levels than the T2D group (*P*<0.01) (Figure 1C). Furthermore, the HOMA-IR index was significantly higher in diabetic rats than in healthy rats (*P*<0.001), while in the T2D+HIIT group, it was lower than in the T2D group (*P*<0.01) (Figure 1D).

Serum levels of lactate and TG

One-way ANOVA showed significant differences among groups in serum lactate ($F_{3,20}$= 112.4, *P*<0.001) and TG ($F_{3,20}$=276.8, *P*<0.001). Tukey's *post hoc* test showed that serum lactate was higher in the T2D group than in the CTL group (*P*<0.05). Additionally, serum TG levels were higher in diabetic rats than in the CTL group (*P*<0.05), while in the T2D+ HIIT group, TG levels were lower than in the T2D group (*P*<0.05) (Figure 2A & B).

Liver expression of GPR81, PPARα, HNF4α and CPT1A expression

Brown-Forsythe analysis showed significant differences among groups for GPR81 ($F_{3,11.63}$=363.0, *P*<0.001), PPARα

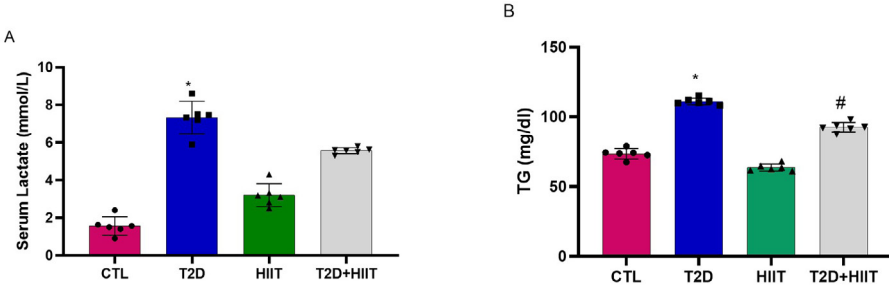


Figure 2. Male wistar rats serum levels of lactate (A) and triglycerides (TG) (B) Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean ± standard error of the mean (n=6), with *P*<0.05 considered statistically significant
CTL: Control, T2D: Type 2 Diabetes, HIIT: High-intensity interval training. * *P*<0.05 vs CTL; # *P*<0.05 vs T2D

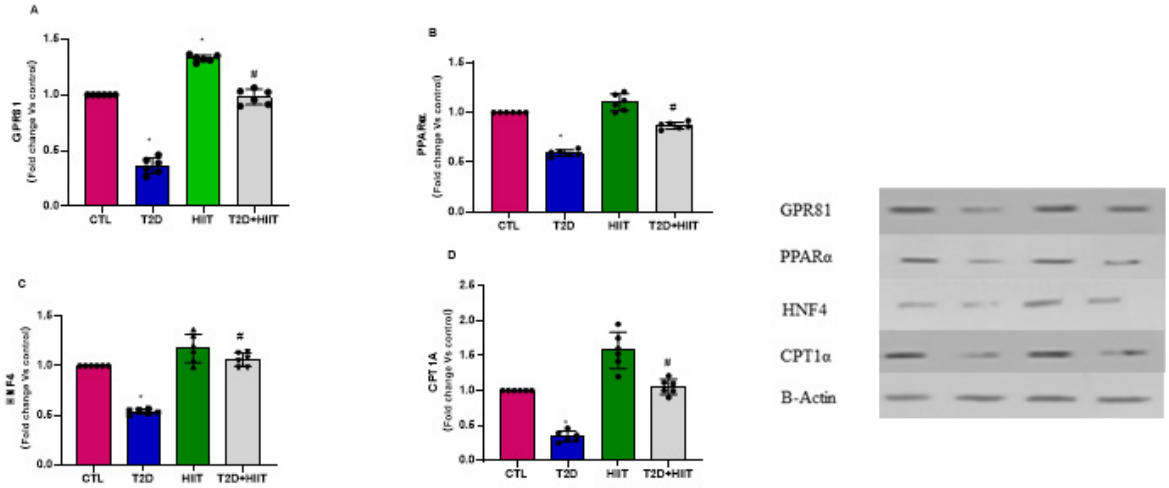


Figure 3. Male wistar rats liver expression of G protein-coupled receptor 81 (GPR81) (A), Peroxisome proliferator-activated receptor alpha (PPARα) (B), Hepatocyte nuclear factor 4 alpha (HNF4A) (C), Carnitine palmitoyltransferase 1A (CPT1A) (D)Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean±standard error of the mean (n=6), with $P<0.05$ considered statistically significant
CTL: Control, T2D: Type 2 Diabetes, HIIT: High-intensity interval training. * $P<0.05$ vs CTL; # $P<0.05$ vs T2D

($F_{3,8.15}$=127.5, $P<0.001$), HNF4α ($F_{3,7.62}$=72.38, $P<0.001$), and CPT1A ($F_{3,7.04}$=88.63, $P<0.001$).

Games-Howell *post hoc* test revealed that GPR81 ($P<0.05$), PPARα ($P<0.05$), HNF4α ($P<0.05$), and CPT1A ($P<0.05$) expression was lower in the liver tissue of the T2D group than in the CTL group. However, in the HIIT+T2D group, GPR81 ($P<0.05$), CPT1A ($P<0.05$), PPARα ($P<0.05$), and HNF4α ($P<0.05$) expression was higher than in the T2D group. No significant difference was observed between the control and HIIT groups (Figure 3A-D).

Hepatic levels of lactate and the expression of MCT1, ACSL4, and EHHADH

Brown-Forsythe and one-way ANOVA showed significant differences among groups in liver expression of MCT1 ($F_{3,11.19}$=164.3, $P<0.001$), ACSL4 ($F_{3,10.41}$=273.4, $P<0.001$), EHHADH ($F_{3,9.31}$=636.7, $P<0.001$), and lactate levels ($F_{3,20}$=68.18, $P<0.01$).

The Games-Howell *post hoc* test showed that liver expression of MCT1 and EHHADH was lower in T2D than in the CTL group ($P<0.05$), but higher in T2D+HIIT than in the T2D group ($P<0.05$). Additionally, the Tukey *post hoc* test showed that liver lactate levels were higher in the HIIT group than in the CTL group and in T2D+HIIT compared with T2D ($P<0.05$). Furthermore, ACSL4 expression was higher in the T2D group than in the CTL and T2D+HIIT groups ($P<0.05$) (Figure 4A-D).

Effects of HIIT on antioxidant enzyme activity in liver tissue following T2D

One-way ANOVA showed that SOD (F3,20=61.21, $P<0.01$), GPX (F3,20=85.63, $P<0.05$), and Catalase

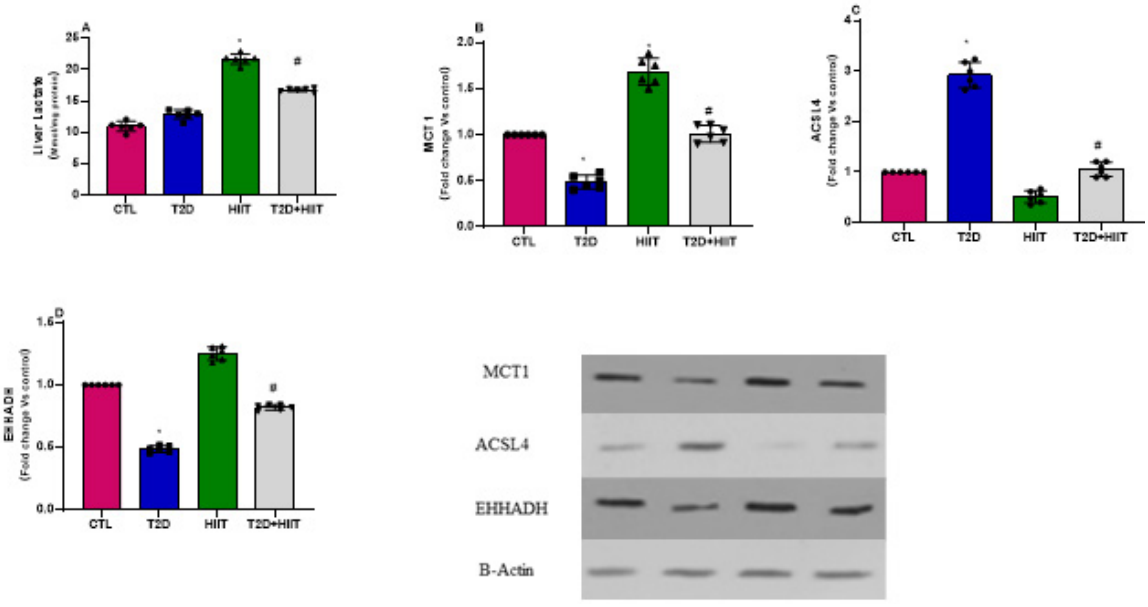


Figure 4. Male Wistar rats expression of lactate (A), Monocarboxylate transporter 1 (MCT1) (B), Acyl-CoA synthetase long chain family member 4 (ACSL4) (C), and enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (EHHADH) (D) Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean±standard error of the mean (n=6), with $P<0.05$ considered statistically significant
CTL: Control, T2D: Type 2 Diabetes, HIIT: High-intensity interval training. * $P<0.05$ vs CTL; # $P<0.05$ vs T2D

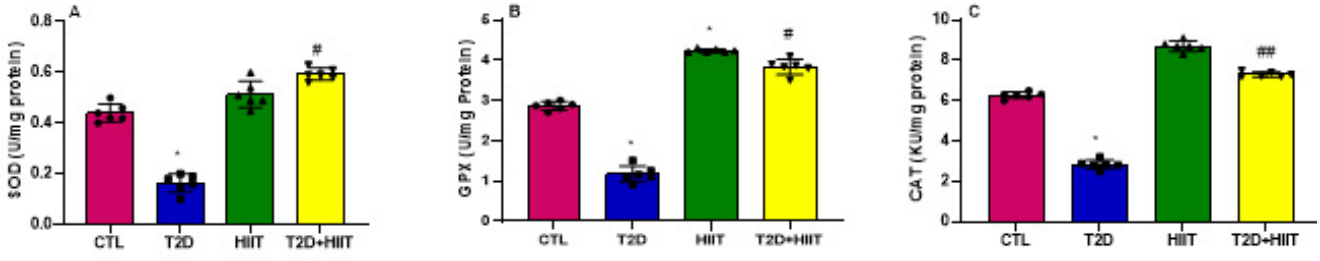


Figure 5. Superoxide dismutase (SOD) (A), glutathione peroxidase (GPX) (B), and catalase (C) activities in liver of male Wistar rats
CTL: Control, T2D: Type 2 Diabetes, HIIT: High-intensity interval training. * P<0.05 vs CTL; # P<0.05 & ## P<0.01 vs T2D

(F3,20=53.58, P<0.05) activities differed significantly among groups. Tukey's *post hoc* test showed that SOD (P<0.05), GPX (P<0.05), and Catalase (P<0.05) activities were reduced in liver tissue due to T2D. However, the T2D+HIIT group showed higher SOD (P<0.05), GPX (P<0.05), and Catalase (P<0.01) activities than the T2D group (Figure 4A-C). Additionally, GPX activity differed significantly between the CTL and HIIT groups (P<0.05) (Figure 5A-C).

Discussion

Our findings suggest that HIIT's ameliorative effects on T2D-induced hepatic injury are associated with enhanced hepatic lactate signaling.

We observed higher serum lactate levels in the T2D group than in the CTL and T2D+HIIT groups. However, hepatic lactate levels were higher in the T2D+HIIT group than in the T2D group. Additionally, T2D reduced hepatic MCT1 levels, whereas HIIT increased hepatic MCT1 expression. Lactate is recognized as a key factor in maintaining metabolic homeostasis (46). Adipose tissue actively produces lactate (47), and under hyperglycemic conditions associated with obesity and T2D, glucose conversion to lactate is enhanced, which could contribute to elevated blood lactate levels (46). In addition, a reduction in hepatic lactate has been reported as a primary cause of insulin resistance (48).

Our study indicates that hepatic MCT1 expression is reduced in T2D, resulting in lower hepatic lactate levels in this group. Consistent with our findings, HIIT has been shown to decrease circulating lactate by reducing hepatic insulin resistance and fat mass (49, 50). Furthermore, aerobic exercise increases both the content and activity of MCT1, thereby facilitating hepatic lactate uptake (32). Similarly, Haj Hosseini *et al.* (51) demonstrated that HIIT increased hepatic MCT1 expression, thereby preventing complications associated with hepatic fat accumulation by facilitating hepatic lactate uptake. This mechanistic interpretation, as well as the rest of the discussion, should be interpreted with caution because the study was not mechanistic in design.

Studies (25, 26) have shown that GPR81, a lactate receptor, plays a crucial role in hepatic tissue lipolysis and maintains hepatic lipid homeostasis. Its expression in the liver significantly increases after fasting, while it decreases in chronic obesity caused by a high-fat diet and T2D (26). Our findings also indicate an association between T2D and reduced GPR81 expression. Additionally, metformin has been reported to improve hepatic injury through mechanisms dependent on GPR81 activation (25). When lactate stimulates GPR81, intracellular adenylate cyclase activity decreases, reducing cAMP production and ultimately suppressing lipolysis. Our results show that performing 8 weeks of HIIT was associated with increased hepatic GPR81 expression. Shang *et al.* demonstrated that lactate improves HIIT-induced hippocampal mitochondrial function through the GPR81-ERK1/2 pathway (52). Additionally, GPR81 plays a significant role during aerobic exercise in regulating intramuscular triglyceride storage and enhancing mitochondrial biogenesis and content via the GPR81–cAMP–PKA pathway (53). Lactate has also been reported to promote cancer cell survival by inhibiting ferroptosis through GPR81 (54).

Our results showed that expression of the GPR81 downstream target, PPARα, was lower in the T2D group than in the CTL and T2D+HIIT groups. PPARα is crucial for fatty acid transport to the mitochondria (55) and for metabolism (6). Increased PPARα expression or activation reduces plasma TG, decreases obesity, and improves hepatic steatosis, thereby enhancing insulin sensitivity (55). PPARα appears to regulate ferroptosis induced by iron overload in the liver. PPARα has been shown to suppress ferroptosis by stimulating expression of glutathione peroxidase 4 (Gpx4) and inhibiting expression of plasma iron transporters (56). Consistent with our study, aerobic training has been shown to improve insulin resistance and hepatic injury in obese rats through increased PPAR-α signaling (57). Additionally, aerobic training has been reported to reduce HFD-induced NASH by increasing hepatic PPARα mRNA expression (58). Beta-oxidation-related genes, including the HNF4α factor, have been identified as potential PPARα targets (7).

Our results showed that T2D reduces hepatic HNF4α expression, whereas HIIT increases it. HNF4α is considered a promising therapeutic target for hepatic injury (59). HNF4α also suppresses ferroptosis. Consistent with our findings, HFD and T2D have been reported to reduce hepatic HNF4α expression (60). Additionally, De Souza *et al.* (61) demonstrated that HIIT can increase HNF4α expression in rat liver tissue.

HNF4α has been shown to increase CPT1A expression, a primary marker of beta-oxidation activity (14). Our findings showed that CPT1A expression decreased in the T2D group, whereas HIIT increased its expression compared with the CTL group. Consistent with our study, CPT1A expression has been reported to be reduced in MAFLD (62). Additionally, decreased fatty acid oxidation due to reduced CPT1A suppression has been shown to exacerbate MAFLD in diabetic animals (62). In contrast, increased CPT1A expression improved fatty acid oxidation, autophagy, glucose homeostasis, and hepatic steatosis (63). Moreover, consistent with our results, other studies have shown that HIIT can increase hepatic CPT1A expression and improve

hepatic function by enhancing fatty acid oxidation (64, 65). Shahouzehi *et al.* (66) reported that HIIT could modulate hepatic glucose metabolism by increasing CPT1 expression.

PPARα, in addition to HNF4α, also induces the enzyme EHHADH (15). Our study found that T2D decreases hepatic EHHADH expression, while HIIT increases it in the liver. Consistent with our findings, an HFD has been reported to reduce EHHADH expression in liver cells (59), and reduced EHHADH expression is associated with increased ferroptosis and enhanced hepatocellular carcinoma (HCC) (67). Aerobic training has been reported to improve insulin resistance and metabolic lipid disorders by increasing PPAR-α expression, which in turn stimulates EHHADH mRNA expression and enhances fatty acid beta-oxidation (68).

Our results showed increased hepatic ACSL4 expression following T2D, whereas HIIT reduced ACSL4 levels in liver cells. ACSL4 has been reported to decrease beta-oxidation by inhibiting PPARα (22). ACSL4 produces lipid peroxides during ferroptosis in beta cells, and increased expression has been observed in rats fed a high-fat diet and in T2D (69). Consistent with our findings, exercise training has been shown to reduce ferroptosis by inhibiting ACSL4 (70). HIIT-induced increases in hepatic lactate appear to stimulate PPARα expression by inhibiting ACSL4, thereby helping regulate ferroptosis. Furthermore, aerobic exercise has been shown to regulate the HFD-induced increase in ACSL, which results in elevated intramuscular fat storage (71).

T2D decreases the activity of antioxidant enzymes in the liver, while HIIT increases their activities. Oxidative stress is both a marker of ferroptosis and a mechanism that induces it (72). The liver is one of the main organs sensitive to hyperglycemia-induced oxidative stress. In diabetes-induced ferroptosis, hepatic levels of CAT, GPX, and SOD decrease (73). Additionally, aerobic training has been reported to improve HFD-induced hepatic injury by increasing CAT levels and inhibiting ferroptosis (74).

Limitations

We should acknowledge two important limitations of this study. First, although animals were randomized before the start of the experiment to reduce allocation bias, diabetic animals were not stratified by FBG or body weight after diabetes confirmation. *Post hoc* comparisons showed comparable baseline FBG and body weight between the T2D and T2D+HIIT groups before training; however, future studies should stratify diabetic animals after induction to better match disease severity. Second, this study did not include an IPGTT or OGTT; therefore, it did not directly assess dynamic glucose handling, and HOMA-IR should be considered only a surrogate marker of insulin resistance. Future studies should incorporate IPGTT/OGTT for more comprehensive metabolic characterization.

Conclusion

Overall, HIIT alleviates T2D–induced hepatic injury by enhancing fatty acid oxidation and reducing ferroptosis-related changes. These beneficial effects are associated with increased hepatic lactate signaling (GPR81), elevated MCT1 expression, and up-regulation of PPARα-dependent pathways and EHHADH following HIIT-induced weight loss.

This study has several limitations to consider when interpreting the findings. First, the sample size in each group (n=6) was relatively small, which may reduce statistical power and increase the risk of type II error. Second, the study focused primarily on protein expression and biochemical markers and did not include histopathological analyses of liver tissue (e.g., steatosis grading, inflammation, fibrosis). Such analyses would strengthen the interpretation of the functional and clinical relevance of the molecular findings. In addition, assessing other key ferroptosis and beta-oxidation markers would help confirm these findings. Third, although the study demonstrated associations between HIIT, lactate/GPR81 signaling, and reduced ACSL (a key player in ferroptosis), it did not establish a direct causal mechanism. The absence of pharmacological inhibition or genetic manipulation (e.g., GPR81 or PPARα knockdown models) limits mechanistic confirmation. In addition, assessment of other key ferroptosis markers is important to confirm ferroptosis. We also assessed only FBG and fasting insulin due to financial limitations. Additional tests, such as OGTT/IPGTT, would provide a more comprehensive assessment of glucose metabolism and insulin sensitivity. STZ itself may cause some degree of liver damage, which should be considered when interpreting these results. However, to minimize this effect, we used a low dose of STZ (30mg/kg) combined with a two-month high-fat diet. Another limitation of this study is the lack of hepatic triglyceride quantification and assessment of additional lipid metabolism markers. Because of resource limitations, these measurements were not included; therefore, the interpretation of hepatic lipid metabolic changes remains incomplete.

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Conflicts of Interest

The authors declare no conflicts of interest

Declaration

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