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CORRESPONDENCE

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Effects of exercise and walnut oil on CES1 and inflammatory factors in the liver of type 2 diabetic rats

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Abstract

Carboxylesterase 1 (CES1) and inflammatory factors play important roles in regulating lipid metabolism and maintaining glucose homeostasis. Exercise and walnut oil have anti-inflammatory effects, but the effects of exercise and walnut oil on CES1 and inflammatory factors in type 2 diabetes mellitus (T2DM) remain to be revealed. 8-week-old SD male rats were randomly divided into normal control group (NC group, $N=12$) and high fat group (HFD group, $N=28$) after 1 week of adaptive feeding. HFD rat models were established by high-fat diet and one-time injection of streptozocin. The successfully constructed rats were randomly divided into diabetes control group (T2DM–NC group, $N=6$), the diabetes plus moderate intensity exercise group (T2DM–MED group, $N=6$), the diabetes plus high intensity intermittent exercise group (T2DM–HIIT group, $N=6$), and the diabetes plus walnut oil group (T2DM–WO group, $N=6$). After 6 weeks of intervention, ELISAs were used to determine the blood glucose and lipid-related indices of the rats. Histomorphologic changes in the liver were observed by hematoxylin–eosin staining. The mRNA expression levels of CES1 and inflammatory cytokines were determined by real-time quantitative PCR. The protein expression levels of CES1 and inflammatory factors were determined by immunofluorescence staining. Compared with those in the NC group, in the T2DM–NC group, FBG, TG, LDL-C and HOMA–IR were increased ($P<0.01$), FINS activity was decreased ($P<0.01$), liver morphology was more disorganized, and CES1 and inflammatory cytokines were highly expressed in the liver tissue ($P<0.05$ or $P<0.01$). After 6 weeks of intervention, TG levels were significantly decreased ($P<0.05$ or $P<0.01$), whereas FINS levels were increased ($P<0.05$ or $P<0.01$), liver morphology was significantly ameliorated, and the CES1, TXNIP and IL-1β expression levels were decreased ($P<0.05$ or $P<0.01$) in the T2DM–MED, T2DM–HIIT and T2DM–WO groups when compared with those in the T2DM–NC group. The T2DM rats presented abnormal blood glucose and lipid levels, and CES1, TXNIP and IL-1β were highly expressed in the liver. Exercise and walnut oil intervention ameliorated the glycolipid metabolism and liver morphology, and reduce the expression of CES1, TXNIP and IL-1β in the liver.

Keywords Exercise, Walnut oil, Type 2 diabetes, CES1, Inflammatory factor

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Introduction

Carboxylesterase 1 (CES1) and inflammatory factors have become important targets for the prevention and treatment of type 2 diabetes mellitus (T2DM) [1]. Studies have suggested that CES1 is a crucial enzyme involved in abnormalities in lipid and glucose metabolism, plays a significant role in lipid homeostasis and is implicated in the pathogenesis of T2DM [2]. Increased expression of CES1 was detected in the livers of T2DM mice [3], and CES1 activity was also significantly increased in T2DM patients [4]. In addition, the levels of several inflammatory factors, such as thioredoxin interacting protein (TXNIP) [5] and interleukin-1β (IL-1β), were found to be elevated in T2DM and involved in the development of T2DM [6]. Studies have shown that hyperglycemia-induced TXNIP overexpression is a typical manifestation of diabetes and insulin resistance (IR), which can activate the NLRP3 inflammasome, thereby releasing IL-1β and IL-18, and leading to pancreatic β-cell apoptosis, cardiomyopathy and metabolic disorders [7]. In T2DM, TXNIP can activate the NLRP3 inflammasome and IL-1β [8, 9]. However, CES1 is also associated with inflammation in the process of regulating energy metabolism. Abozaid et al. reported that CES1 and inflammation are involved in the pathogenesis of fatty liver disease and fibrosis [10]. Nevertheless, the relationships between CES1 and inflammatory factors such as TXNIP and IL-1β and the mechanism of action of these inflammatory factors in diabetes still need to be further explored.

As an economical and effective nondrug therapy, exercise can inhibit the expression of CES1 [11], TXNIP, IL-1β [12] and other inflammatory factors, which has become an important means of clinical prevention and treatment of diabetes. Our preliminary results revealed that different exercise modes could reduce the expression levels of CES1 and inflammatory factors in the skeletal muscle of T2DM rats [11]. Alec et al. reported that aerobic exercise resulted in a sharp decrease in TXNIP expression [13]. Li et al. reported that different exercise intensities could downregulate the expression of TXNIP and related factors, thereby protecting the heart from injury, and that moderate intensity exercise had the best protective effect [14]. Previous studies have shown that 12 weeks of Tai Chi exercise can inhibit the expression of IL-1β and related factors to maintain blood glucose homeostasis in the serum of prediabetic individuals [15]. Karstoft et al. also reported that regular exercise reduces the levels of C-reactive protein (CRP), IL-1β and tumor necrosis factor (TNF-α), and increases the level of anti-inflammatory factors, which serves as an ideal means to inhibit the inflammatory response and ameliorate diabetes mellitus [16]. Furthermore, walnut oil also has anti-inflammatory effects. Studies have indicated that walnut oil has antioxidant and anti-inflammatory effects, which can ameliorate pathological morphology, reduce the production of reactive oxygen species (ROS) and the release of proinflammatory cytokines, and downregulate the expression of the NLRP3 inflammasome [17], thereby inhibiting the production of inflammatory factors and enhancing its antioxidant capacity [18, 19]. Miao et al. reported that walnut oil can reduce serum TNF-α and IL-1β levels, and protect mice against LPS-induced acute intestinal injury [20]. These studies indicate that exercise and walnut oil have anti-inflammatory effects, but the effects of exercise and walnut oil on CES1 and other inflammatory factors in T2DM rats have not been fully elucidated.

Therefore, we established a T2DM rat model, exercise and walnut oil interventions were adopted, and an ELISA detection method, hematoxylin–eosin staining, real-time quantitative PCR, and immunofluorescence staining were used to explore the effects of exercise and walnut oil intervention on CES1 and inflammatory factors in T2DM rats to provide a potential treatment option for patients with diabetes.

Materials and methods

Experimental animals

8-week-old male Sprague–Dawley (SD) rats were purchased from Wuhan Animal Model Research Center (Production license No: SCXK 2022-0012; Number: 40; Initial body weight: 219.00 ± 9.67 g), and was feeded in the Animal Center of Yangtze University. Adaptive feeding for 1 week was randomly divided into two groups using a random number table: normal control group (NC group, $N=12$) and high fat group (HFD group, $N=28$). The rats in the NC group were given ordinary diet, and the rats in the HFD group were given high-fat diet with 45% fat, and weighed weekly. After 8 weeks of high-fat feeding, rats in the HFD group were injected with once streptozotocin (STZ) (30 mg/kg) intraperitoneally for modeling. The successfully constructed rats were randomly divided into diabetes control group (T2DM–NC group, $N=6$), diabetes plus moderate intensity exercise group (T2DM–MED group, $N=6$), diabetes plus high intensity intermittent exercise group (T2DM–HIIT group, $N=6$), and diabetes plus walnut oil group (T2DM–WO group, $N=6$). The experimental scheme was approved by the animal Experiment Ethics Review Committee of Yangtze University (NO.2022120044).

Main reagents

Total RNA extraction reagent (Trizol purchased from Novizan Biotechnology Company); Reverse transcription kit and real-time fluorescence quantification kit (TaKaRa); β-actin primer (AuGCT); Target gene primer

(Tianyi Huiyuan); β-actin antibody (Cell Signaling); TXNIP antibody (Wanlei); CES1 antibody (Zenbioscience); IL-1β antibody (Proteintech); Streptozotocin (Beijing Boai Port Company); TYR-488 (Pinofil Bio); TYR-555 (Pinofil Bio); Tris–EDTA Repair Solution 20* (Pinofil Bio); Citric Acid Repair Solution 20* (Pinofil Bio); HRP–anti-rabbit IgG (Pinofil Bio); Triton X-100 (Beijing Solaibao Technology Co., LTD).

Main instruments

Swirl mixer (Jintan Jinyi Medical Instrument Factory); ultra-clean workbench (Beijing Donglian Hal Instrument manufacturing Company); ultrapure water meter (ELGA Company); Benchtop high speed refrigerated microcentrifuge (Thermo Company); 37℃ constant temperature incubator (Taicang Huamei Biochemical Instrument); constant temperature metal bath (Shanghai Zhida Instrument); fluorescent quantitative PCR (ThermoFisher Scientific); ultra-pure water system (MiniQ Company); PCR amplification instrument (Applied Biosystems); general optical microscope (Leica Company); electrophoresis apparatus (Bio-Rad Company); ultra-low temperature −80℃ refrigerator (Haier Company); rat running table (Anhui Zhenghua Company); imaging System (Bio-Rad Company).

Walnut oil intervention

Walnut oil is Dabu wild walnut oil (NO.GPXVT-S6O124065FE, Shannan City, Xizang Province, China), provided by the Tibet Plateau Walnut Industry Research Institute of Yangtze University. The intragastric dose was 1.2 g/kg, and the intervention time was 5:00–6:00 PM from Monday to Friday for a total of 6 weeks.

Treadmill exercise training

The T2DM–MED and T2DM–HIIT groups underwent adaptive exercise for 1 week, 30 min a day for 5 days. The exercise intensity of the T2DM–MED group was 10 m/min for the first 3 days and 12 m/min and 14 m/min for the second 2 days, respectively. The exercise intensity of the T2DM–HIIT group was 12 m/min for the first 3 days, and 14 m/min and 18 m/min for the second 2 days, respectively. The formal exercise was treadmill exercise for 6 weeks, and the exercise intensity in the T2DM–MED group was 12 m/min (increased by 2 m/min per week for the first 2 weeks, and remained unchanged at 16 m/min for the last 3 weeks, equivalent to 60% maximal oxygen intake [21]), and the slope was 0°. The exercise intensity of T2DM–HIIT group was 16 m/min (7 min) × 1 group (rest for 30 s), 20 m/min (3 min × 6 groups) (rest for 30 s per group), 15 m/min (3 min × 6 groups) (rest for 30 s per group). 16 m/min (7 min) × 1 group (equivalent to 70% VO_2 Max [21]) with a slope of 0°. 50 min a day, 5 days/week (rest on Saturday and Sunday), the intervention time was 5:00–6:00 PM on the training day. The rats in the T2DM group maintained a high fat diet during the intervention period.

Sample collection

After training, the rats were fasted for 12 h, anesthetized with isoflurane, and decapitated quickly after orbital blood extraction. The blood was left at room temperature for 30 min, and then centrifuged, after which the supernatant was collected for use. The liver was quickly extracted; part of it was put into a fixed solution (for morphological detection), and part of it was immediately frozen in liquid nitrogen and then transferred to a freezer at −80 °C for storage for measurement.

Hematoxylin–eosin staining

The liver tissues were fixed in 4% paraformaldehyde for 24–48 h. After the liver tissue was fixed, it was rinsed, dehydrated, cleared, encased, sliced, and dried. The slices were dewaxed and washed, followed by treatment with dye solution 1 (main component: hematoxylin), dye solution 2 (main component: differentiation solution), dye solution 3 (main component: blue return solution), 85% ethanol, 95% ethanol, dye solution 4 (main component: eosin), anhydrous ethanol I, anhydrous ethanol II, anhydrous ethanol III, n-butanol, xylene I, and xylene II. The samples were quickly blown dry, sealed, observed, and imaged with an Olympus BX51 optical microscope. A minimum of 3 images per section (3 sections per slide), 1 slide per animal, and 3 animals per exposure group were used for analysis. Images were analyzed by Image J.

Real-time fluorescence quantitative PCR detection

Total RNA was extracted from the liver tissue using TRIzol (R411-01, Vazyme). The impurity was removed by chloroform extraction, the RNA was recovered by precipitation, and the purity and concentration of RNA were determined by Nanodrop method. A reverse transcription kit (TAKARA, RR047A) was used to remove DNA impurities and reverse transcribed RNA into cDNA (removal of DNA: 42 °C, 2 min, 4 °C, 1 min; Reverse transcription: 37 °C, 15 min; 85 °C, 5 s). Subsequently, the relative expression of the target gene was detected using a fluorescence quantitative kit (TAKARA, RR420A) and fluorescence quantitative PCR (ABI7500, ThermoFisher Scientific) (reaction condition was 95 °C, 30 s predenatured; 95 °C, 5 s; 60 °C, 34 s, 40 cycles; 95 °C, 15 s, 60 °C, 1 min; 95 °C, 15 s). Target gene primers (CES1, TXNIP, and IL-1β) and internal reference primers (β-actin) were added for detection. The relative expression levels of CES1, TXNIP and IL-1β were calculated by $2^{-\triangle\triangle}$Ct. The primers used are shown in Table 1.

Table 1 Sequence of gene expression primers determined by real-time PCR

Target genes		Genetic sequences
CES1	Forward primer 5′–3′	TCCCGTGGACATAGAGATGCTG
	Reverse primer 5′–3′	CTCCATGATCTCCTACCACCGT
TXNIP	Forward primer 5′–3′	CCACGCTGACTTTGAGAACA
	Reverse primer 5′–3′	GGAGCCAGGGACACTAACATA
IL-1β	Forward primer 5′–3′	GCACAGTTCCCCAACTGGTA
	Reverse primer 5′–3′	ACACGGGTTCCATGGTGAAG
β-Actin	Forward primer 5′–3′	GCTCTGGCTCCTAGCACCAT
	Reverse primer 5′–3′	GCCACCGATCCACACAGAGT

Immunofluorescence staining analysis

The slices were soaked in xylene and anhydrous ethanol, rinsed, repaired at high temperature and high pressure, sealed, and incubated. The primary antibody was diluted with an antibody: diluent ratio of 1:200 (or other appropriate dilution ratios) such as CES1: Zenbioscience, R382215, dilution ratio: 1:200, Fluorescein (TSA): TYR-555 (IF555); TXNIP: Wanlei, WL05902, dilution ratio: 1:100, Fluorescein (TSA): TYR-488 (IF488); IL-1β: Proteintech, 26048-1-AP, dilution ratio of 1:200, Fluorescein (TSA): TYR-488 (IF488), and incubated overnight at 4 °C or for 2 h at 37 °C. The secondary antibody was diluted with PBST and incubated with the tissue. DAPI working droplets were added to the tissue, followed by incubation and washing. The liquid was dried, an anti-fluorescent sealer was added, and the film was sealed and stored in the dark. Fluorescence was excited at a specific wavelength, observed and recorded by fluorescence microscopy (ECLIPSE Ci, Nikon), and data was processed by Image J and SPSS 23.0 software. The scale was 100 μm and the magnification was 200 times. The maximum excitation wavelength of TYR-488 was 490 nm, the maximum emission wavelength was 520 nm, and the color was green. The maximum excitation wavelength of TYR-555 was 550 nm, the maximum emission wavelength was 570 nm, and the color was red. The maximum excitation wavelength of DAPI was 345 nm, the maximum emission wavelength was 455 nm, and the color was blue.

Main observation index

① After 6 weeks of intervention, the expression of blood glucose, insulin resistance, and blood lipids in each group was observed.

② Liver histomorphological changes in rats.

③ The mRNA and protein expression levels of CES1, TXNIP, and IL-1β in the rat liver.

Statistical analysis

All experimental data were analyzed and processed by SPSS 23.0 software, and were expressed as mean ± standard deviation (M ± SD). One-way analysis of variance was used for comparison among the groups. $P < 0.05$ indicates a significant difference. Histograms are plotted by GraphPad Prism 8.0 software.

Results

Successful establishment of T2DM model

After 8 weeks of high-fat feeding, the rats in the HFD group were injected with STZ (30 mg/kg) intraperitoneally for modeling. Fasting blood glucose (FBG) was measured 3 days and 1 week after injection. If the FBG level was > 11.1 mmol/l and glucose tolerance was impaired, T2DM was considered to be successfully induced in the rats [22]. As shown in Fig. 1A, body weight in the T2DM group was greater than that in the NC group only at week 8 ($P < 0.05$), and there was no significant difference at other timepoints ($P > 0.05$). However, the blood glucose level was significantly greater than that in the NC group ($P < 0.01$) (Fig. 1B). In the glucose tolerance test, the blood glucose levels of the rats in the T2DM group at 0, 30, 60, 90 and 120 min were significantly greater than those in the NC group ($P < 0.01$ or $P < 0.05$); that is, the glucose tolerance of the rats in the T2DM group was reduced or impaired. Moreover, the area under the glucose tolerance curve of the rats in the T2DM group was significantly greater than that in the NC group, indicating impaired glucose metabolism and reduced insulin sensitivity (Fig. 1C, D). In conclusion, FBG was shown to be greater than 11.1 mmol/l and glucose tolerance were reduced or impaired, indicating the successful establishment of a T2DM rat model. The successfully induced T2DM rats were randomly divided into the T2DM–NC, T2DM–MED, T2DM–HIIT, and T2DM–WO groups.

Effects of different interventions on body weight, glycolipid metabolism and IR in T2DM rats

Throughout the intervention period, the body weights of the T2DM–NC, T2DM–MED, T2DM–HIIT and T2DM–WO groups were lower than those of the NC group (Fig. 2). As shown in Fig. 3 and Table 2, the results of glucose and lipid metabolism revealed that FBG, triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and homeostasis model assessment–insulin resistance (HOMA–IR) were increased in the T2DM–NC group compared with those in the NC group ($P < 0.01$), whereas fasting insulin (FINS) activity was decreased ($P < 0.01$). After 6 weeks of intervention, compared with those in the T2DM–NC group, in the T2DM–MED group, TG,

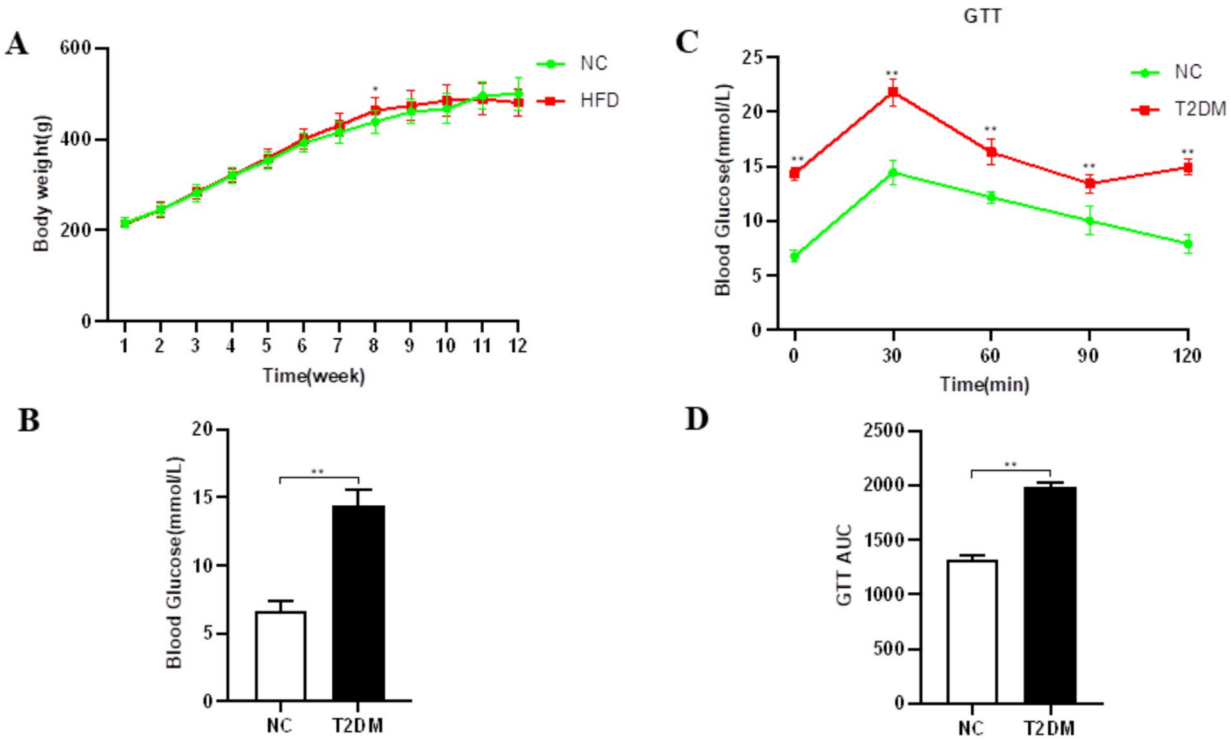

Fig. 1 Successful establishment of the T2DM rats model. **A** Changes in body weight during the high-fat diet. **B** Curve of glucose tolerance test in the T2DM and NC groups. **C** Fasting blood glucose in the T2DM and NC groups. **D** Area under the curve of the glucose tolerance in the T2DM and NC groups. $^{*}P<0.05$, $^{**}P<0.01$ vs. NC

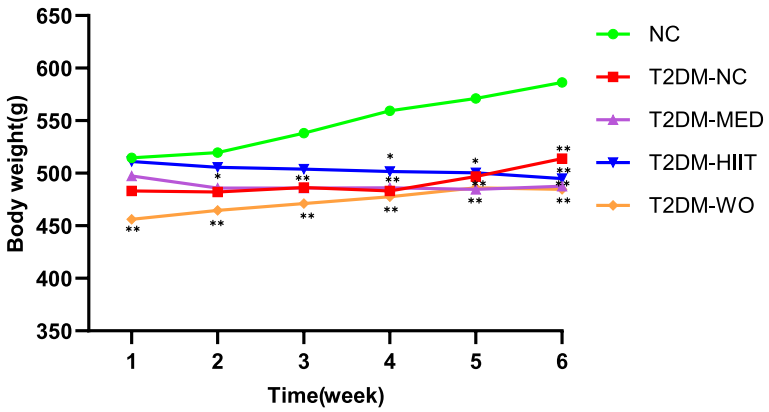

Fig. 2 Changes in body weight of rats in each group. Changes in body weight of different groups during the intervention, $^{*}P<0.05$, $^{**}P<0.01$ vs. NC

LDL-C and HOMA–IR were significantly lower ($P<0.01$), and FINS was greater ($P<0.05$), whereas in the T2DM–HIIT group, TG and LDL-C were significantly lower ($P<0.01$), and FINS was significantly greater ($P<0.01$), and in the T2DM–WO group, TG was lower ($P<0.05$), and FINS was greater ($P<0.05$). FINS and HOMA–IR were significantly greater in the T2DM–HIIT group than in the T2DM–MED group ($P<0.01$). Compared with that in the T2DM–HIIT group, TG was significantly greater in the T2DM–WO group ($P<0.05$), and FINS was significantly lower ($P<0.01$).

Effects of different interventions on the liver morphology in rats

As shown in Fig. 4, the results of the liver hematoxylin–eosin staining revealed that the liver lobules were complete in shape and clear in structure, the nuclei were uniform in size, the liver cells were arranged neatly, the

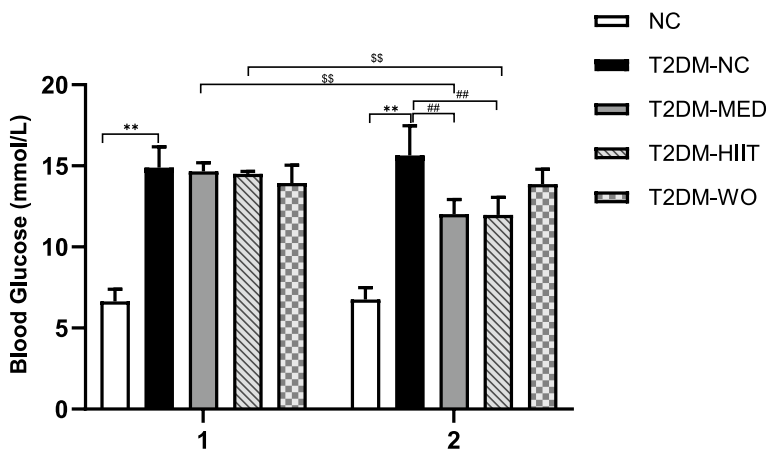

Fig. 3 Changes in blood glucose before and after intervention. 1 indicates before intervention, 2 indicates after intervention. Blood glucose concentration (mmol/L) in the normal control group (NC = 12), the diabetes control group (T2DM–NC = 6), the diabetes plus moderate intensity exercise group (T2DM–MED = 6), the diabetes plus high intensity intermittent exercise group (T2DM–HIIT = 6), and diabetes plus walnut oil group (T2DM–WO = 6), respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. T2DM–NC; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. before

Table 2 Changes in blood lipid and other related indexes in different groups after intervention

Index	NC (*N* = 12)	T2DM–NC (*N* = 6)	T2DM–MED (*N* = 6)	T2DM–HIIT (*N* = 6)	T2DM–WO (*N* = 6)
TG (mmol/l)	0.93 ± 0.36	1.96 ± 0.73**	0.83 ± 0.31$^{\#\#}$	0.61 ± 0.28$^{\#\#}$	1.37 ± 0.46$^{**\#\$}$
TC (mmol/l)	2.08 ± 0.24	2.23 ± 0.32	1.98 ± 0.33	1.85 ± 0.56	1.95 ± 0.30
HDL-C (mmol/l)	1.20 ± 0.12	1.09 ± 0.43	1.29 ± 0.14	1.17 ± 0.43	1.07 ± 0.14
LDL-C (mmol/l)	0.40 ± 0.07	0.54 ± 0.13**	0.37 ± 0.10$^{\#\#}$	0.34 ± 0.10$^{\#\#}$	0.45 ± 0.09
FINS (mIU/L)	16.56 ± 0.95	13.94 ± 0.40**	15.05 ± 0.96$^{\#}$	17.04 ± 0.84$^{**\#\#\&\&}$	14.89 ± 0.75$^{**\#\$\$}$
HOMA–IR	4.98 ± 0.71	9.70 ± 1.27**	8.03 ± 0.49$^{**\#\#}$	9.07 ± 0.91$^{**\&\&}$	9.19 ± 0.74**

$^{**}P < 0.01$ vs. NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. T2DM–NC; $^{\&\&}P < 0.01$ vs. T2DM–MED; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. T2DM–HIIT

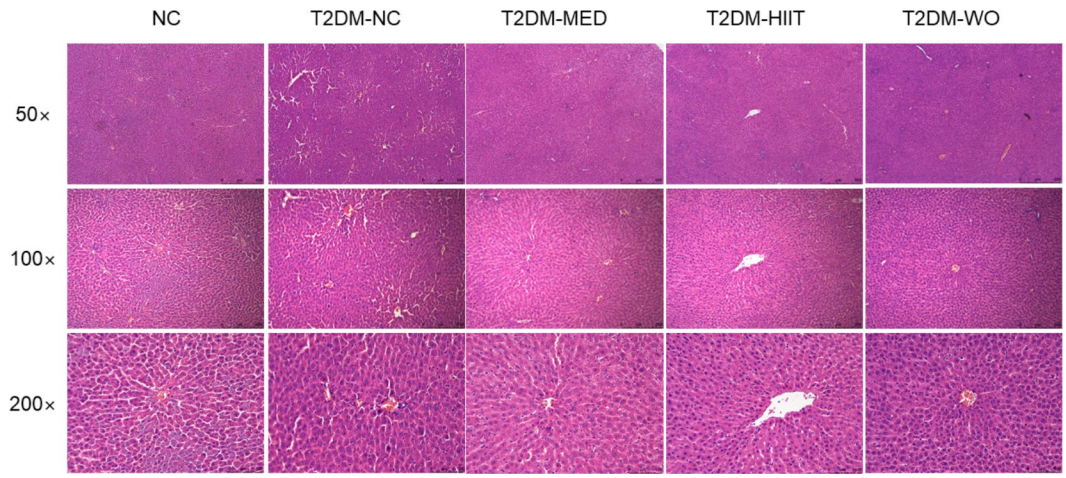

Fig. 4 Changes in liver morphology after intervention in rats. Representative images of paraffin-embedded sections staining with hematoxylin–eosin demonstrating changes in the liver morphology after intervention in the NC, T2DM–NC, T2DM–MED, T2DM–HIIT and T2DM–WO groups. *N* = 3 rats/group, the scales are 500 μm, 250 μm and 100 μm, respectively, and the magnifications are 50×, 100× and 200×

liver cords were arranged radially and regularly, and there was no vacuolar degeneration in the NC group. In the T2DM–NC group, the structure of the hepatic lobules was disordered, the boundary was not clear, fat vacuoles could be observed, the arrangement of hepatocytes was disordered, and the hepatic blood sinuses were dilated. Compared with those in the T2DM–NC group, in the T2DM–MED, T2DM–HIIT and T2DM–WO groups, the hepatic lobular structure and cord structure of hepatocytes were more clearly arranged, there were no significant fat vacuoles, and hepatic blood sinuses were narrow, suggesting that exercise and walnut oil intervention improved on liver morphology.

Changes in the liver mRNA levels of CES1 and inflammatory cytokine under different interventions

To further determine the mRNA expression of CES1, TXNIP and IL-1β in the liver and the role of intervention in T2DM rats, qRT-PCR was subsequently performed. As shown in Fig. 5, the qRT-PCR results revealed that the mRNA expression levels of CES1, TXNIP and IL-1β in the T2DM–NC group were significantly greater ($P<0.01$) than those in the NC group. Compared with those in the T2DM–NC group, the mRNA expression levels of CES1, TXNIP and IL-1β in the T2DM–MED, T2DM–HIIT and T2DM–WO groups were decreased to a certain extent ($P<0.01$). In addition, there were no significant differences in the CES1, TXNIP or IL-1β mRNA expression levels in the T2DM–MED and T2DM–HIIT groups when compared with the T2DM–WO group ($P>0.05$).

Changes of liver protein expression of CES1 and inflammatory cytokine under different interventions

The results of immunofluorescence staining and quantitative analysis revealed that compared with those in the NC group, the numbers of CES1, TXNIP and IL-1β positive cells were markedly greater, and the average fluorescence intensities of CES1, TXNIP and IL-1β were greater in the livers of the T2DM–NC group ($P<0.01$ or $P<0.05$). Under the microscope, the fluorescence intensities of CES1, TXNIP and IL-1β in the NC group were lower, whereas the fluorescence intensities of CES1, TXNIP and IL-1β in the T2DM–NC group were greater, and the protein expression levels of CES1, TXNIP and IL-1β were significantly increased. These results indicate that the expression levels of CES1, TXNIP and IL-1β in the liver tissue of diabetic rats significantly increased and that the rats were in an inflammatory state. As shown in Fig. 6, compared with those in the T2DM–NC group, the contents of CES1, TXNIP, and IL-1β were significantly lower,

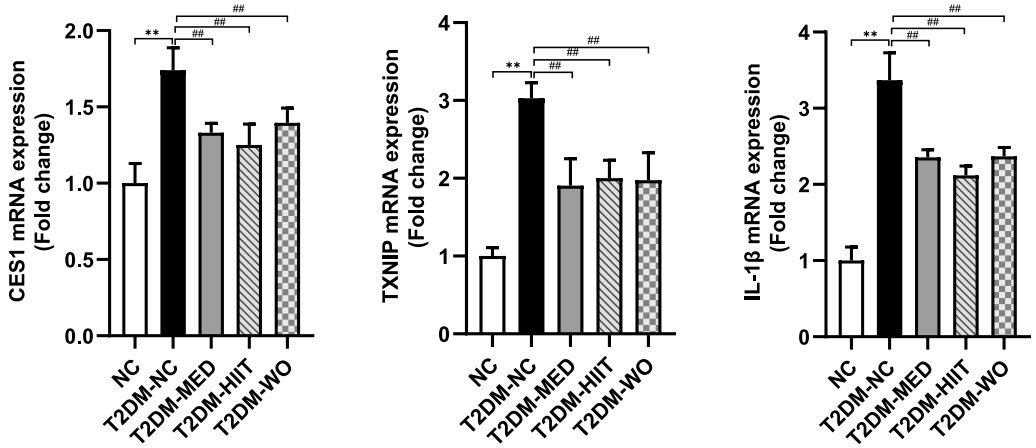

Fig. 5 Changes of mRNA levels of CES1 and inflammatory factors in the liver of rats after intervention. Relative mRNA levels of CES1, TXNIP, and IL-1β in the livers of rats in the NC, T2DM–NC, T2DM–MED, T2DM–HIIT and T2DM–WO groups, respectively ($N=3$); $^{*}P<0.05$, $^{**}P<0.01$ vs. NC; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. T2DM–NC

(See figure on next page.)

Fig. 6 Immunofluorescence analysis of CES1 and inflammatory factors expression in the rat liver. **A** Immunofluorescence staining of CES1 and TXNIP in the liver tissue ($N=3$). The red color represents areas of CES1, the green color represents areas of TXNIP, and the blue color represents the nucleus. The scale is 100 μm and the magnification is 200 times. **B** Quantitative analysis of the relative expression levels of CES1 and TXNIP protein in (**A**). **C** Immunofluorescence staining of CES1 and IL-1β in the liver tissue ($N=3$). The red color represents areas of CES1, the green color represents areas of IL-1β, and the blue color represents the nucleus. **D** Quantitative analysis of the relative expression levels of CES1 and IL-1β protein in (**C**). The scale is 100 μm and the magnification is 200 times. $^{*}P<0.05$, $^{**}P<0.01$ vs. NC; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. T2DM–NC; $^{\&}P<0.05$, $^{\&\&}P<0.01$ vs. T2DM–MED; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs. T2DM–HIIT

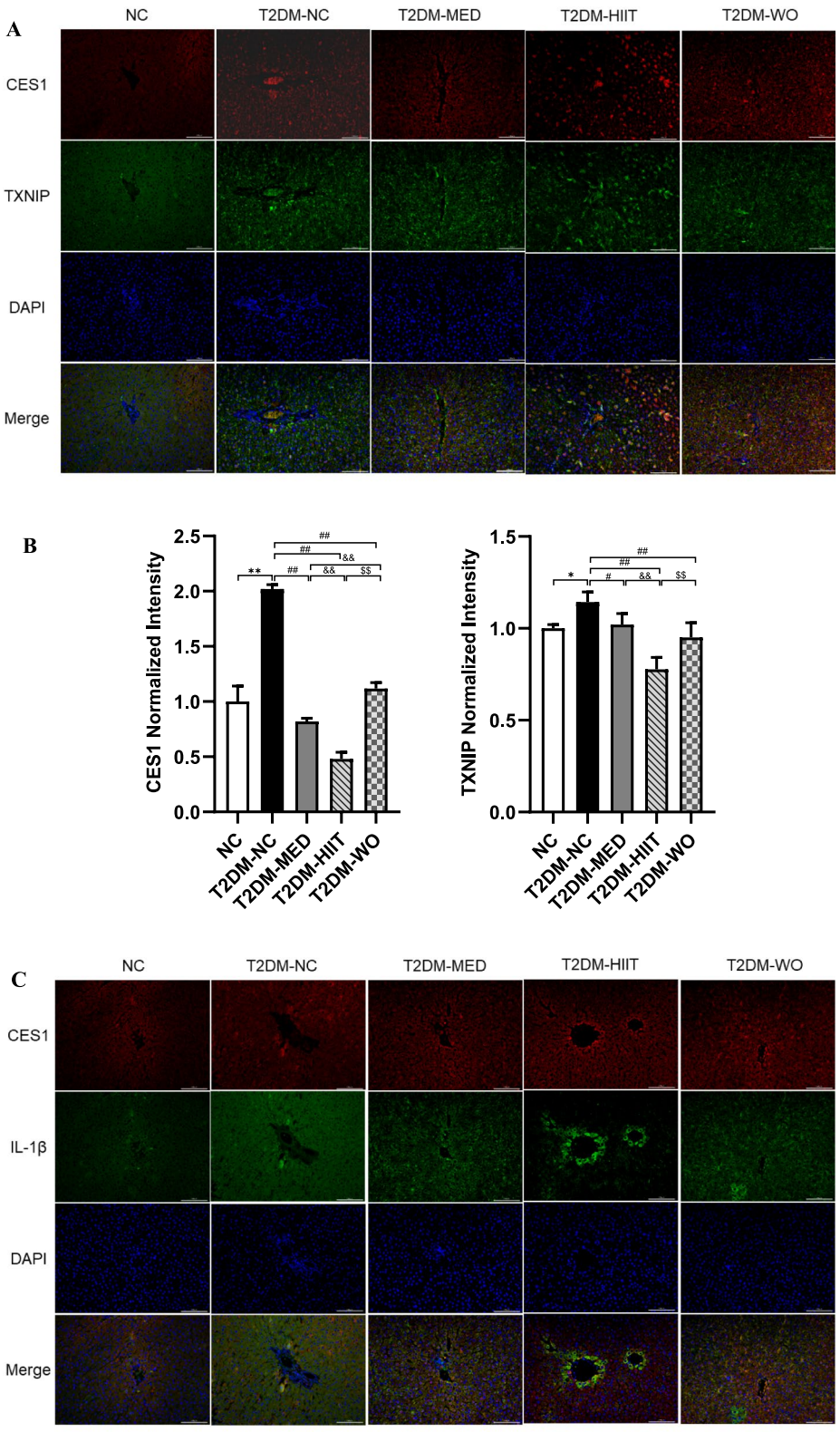

Fig. 6 (See legend on previous page.)

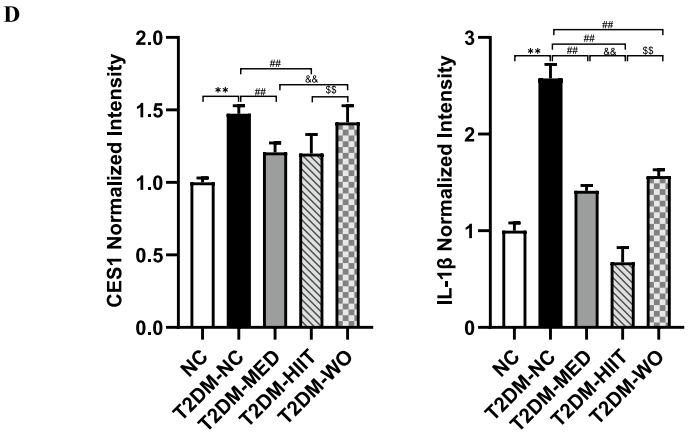

Fig. 6 continued

and the CES1, TXNIP, and IL-1β expression levels were significantly lower in the T2DM–MED, T2DM–HIIT, and T2DM–WO groups. The average fluorescence intensity of CES1, TXNIP, and IL-1β was significantly lower ($P < 0.01$ or $P < 0.05$) in the T2DM–MED, T2DM–HIIT, and T2DM–WO groups than in the T2DM–NC group, suggesting that exercise and walnut oil may reduce the expression of CES1, TXNIP, and IL-1β.

Discussion

A T2DM rat model with IR and β-cell damage was established by combining intervention with a high-fat diet and STZ. One study also revealed that a HFD combined with STZ was a reasonable animal model for T2DM [23]. We found increased FBG and impaired glucose tolerance, suggesting that the T2DM model was successfully established. Furthermore, our data revealed that body weight was not significantly different between the NC group and the T2DM–NC group before intervention, which may be related to the low fat content in the high-fat feed. After STZ injection, the body weight of the T2DM–NC group was significantly lower than that of the NC group, which may be related to STZ drug intolerance. After the intervention, the weight of the T2DM–NC group was consistently lower than that of the NC group, which was associated with weight loss caused by diabetes.

The role of inflammation in diabetes and IR has received increasing attention and has become a potential target for the prevention and treatment of diabetes and metabolic diseases [24]. When the T2DM rat model was established, changes in glucose and lipid metabolism, IR and liver morphology were analyzed. Compared with those in the NC group, FBG, TG, LDL-C and HOMA–IR were increased, FINS activity was decreased, the hepatic lobular structure was disrupted, adipose vacuoles were significant, the hepatocyte arrangement was disordered, and the hepatic sinusoid was dilated in the T2DM–NC group, suggesting that T2DM rats presented abnormal glucose and lipid metabolism, increased IR and disordered liver morphology. After 6 weeks of intervention, FBG, TG and LDL-C significantly decreased, FINS increased, and liver morphology improved in all exercise groups, indicating that exercise improved glucose and lipid metabolism and liver morphology and increased FINS content. In the T2DM–WO group, TG decreased, FINS increased and liver morphology was ameliorated after intervention, suggesting that walnut oil can ameliorate TG and liver morphology, and increase FINS.

CES1 activity is closely associated with metabolic disorders [25], regulates lipid metabolism [3], and is highly expressed in diabetic mice [26, 27]. Studies have shown that CES1 expression in vivo and in vitro can be induced by glucose [3], which is closely related to IR [28]. The results of this study revealed that the CES1 mRNA level and positive expression of the CES1 protein in the livers of T2DM rats increased significantly, indicating glucose and lipid metabolism disorders in the liver tissues of T2DM rats. Previous studies have shown that the expression level of CES1 in the skeletal muscle tissue of T2DM rats decreases significantly after exercise [11]. The results of this study revealed that exercise significantly reduced CES1 mRNA expression level and positive protein expression in the liver tissue of T2DM rats, suggesting that exercise could reduce CES1 expression in the liver tissue of T2DM rats. However, few studies have investigated the correlation between walnut oil and CES1 expression in T2DM rats. Our experimental data revealed that walnut oil can decrease CES1 mRNA expression and positive protein expression in the liver tissue of T2DM rats, indicating that walnut oil has a certain effect on reducing the expression of CES1. TXNIP is sensitive to glucose responses in pancreatic beta cells [29], and plays

an important role in triggering NLRP3 inflammasome activation and IL-1β secretion [5, 9]. Inhibition of TXNIP was found to attenuate the glucose-induced activation of NLRP3 in pancreatic beta cells [30]. IL-1β is a key component of chronic inflammatory responses [31] and an important inflammatory mediator in T2DM and IR [32]. Studies have shown that T2DM patients have increased levels of IL-1β, which is involved in the development of T2DM and IR [33]. In addition, a high glucose concentration supplies the priming signal for the transcription of IL-1β by the activation of TXNIP, which subsequently facilitates increased IL-1β expression levels [33]. The experimental data revealed that the mRNA expression levels of TXNIP and IL-1β were significantly increased in the livers of T2DM rats, and the number of positive cells and positive protein expression of TXNIP and IL-1β were significantly increased, indicating that the livers of T2DM rats were in a state of chronic inflammation. In the animal and clinical experiments, exercise has been shown to reduce the expression levels of TXNIP and IL-1β and ameliorate the inflammatory state. For example, Cai et al. reported that aerobic exercise training inhibited apoptosis and improved cardiac fibrosis and cardiac function in mice after myocardial infarction by downregulating the TXNIP expression and ROS levels [34]. Hong et al. reported that exercise alleviated atherosclerotic coronary endothelial dysfunction by reducing TXNIP/NLRP3 inflammasome activity and oxidative stress [35]. Rose et al. reported that 4 week treadmill exercise significantly reduced the TXNIP content in the hippocampus of mice and alleviated inflammation [36]. Li et al. reported that 4 week treadmill exercise could reduce the levels of the NLRP3 inflammasome and IL-1β in diabetic rats, inhibit the NLRP3/IL-1β signaling pathway, and thus reduce the inflammatory state [37]. Tomeleri et al. showed that 12 weeks of resistance exercise can reduce the levels of proinflammatory factors such as IL-1β in elderly women with T2DM [38]. In addition, studies have demonstrated that walnut oil has anti-inflammatory, antitumor, and antioxidative effects, and plays important roles in neuroprotection, cardiac protection, and immune regulation [39]. Walnut oil has anti-inflammatory effects on LPS-induced cell damage by inhibiting the activation of the toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor kappa-B (NF-κB) pathway [19]. Walnut oil also downregulates the expression of the NLRP3/ASC/capase-1 inflammatory pathway by reducing the production of ROS and the release of proinflammatory cytokines [17]. Miao et al. reported that walnut oil can reduce the levels of serum TNF-α and IL-1β, and has a protective effect on LPS-induced acute intestinal injury in mice [20]. Our data show that moderate intensity aerobic exercise, high intensity intermittent (HIIT) and walnut oil can significantly reduce the mRNA and protein expression of TXNIP and IL-1β in the livers of diabetic rats, suggesting that exercise and walnut oil can reduce the expression levels of inflammatory factors in the livers of diabetic rats. However, there was no significant difference between the exercise and walnut oil intervention groups, and the exercise plus walnut oil group was not included in the experiment. The advantages and disadvantages of different intervention methods will be further clarified in subsequent experiments.

In addition, CES1 is closely related to inflammatory factors. A previous study revealed that CES1 and IL-18 expression levels are increased in fatty liver disease models and are involved in the pathogenesis of fatty liver disease and fibrosis [10]. Phillips et al. reported that CES1 regulates the production of IL-1β in macrophages [40]. Quantitative immunofluorescence analysis also revealed that the positive expression of CES1, TXNIP, CES1 and IL-1β was upregulated in the livers of T2DM rats simultaneously, indicating that CES1, TXNIP, and IL-1β were highly expressed in the livers of T2DM rats and closely related. However, the upstream and downstream relationships among CES1, TXNIP, and IL-1β need to be further verified.

Conclusion

The results of this study indicated that the T2DM rats were in a state of abnormal glycolipid metabolism and that CES1, TXNIP, and IL-1β were highly expressed in the liver. Exercise and walnut oil led to statistically significant decreases in TG, CES1, TXNIP, and IL-1β levels and generally improved glycolipid metabolism and inflammatory states. However, the limitations of this study are as follows. First, a walnut oil plus exercise group was not included, and the specific effects of the two different interventions on inflammatory factors in diabetic rats are not clear. Second, the expression of NLRP3, ASC, and caspase-1, the release of the proinflammatory cytokine IL-18, and the degree of cellular inflammatory infiltration were not assessed in this study. Third, the anti-inflammatory effect of walnut oil on oxidative stress-related signals is also very interesting and should be further explored in follow-up research.

Abbreviations

CES1	Carboxylesterase 1
T2DM	Type 2 diabetes mellitus
TXNIP	Thioredoxin interacting protein
IL-1β	Interleukin-1β
IR	Insulin resistance
CRP	C-reactive protein
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α
SD	Sprague–Dawley
FBG	Fasting blood glucose
TG	Triglyceride

LDL-C	Low density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment–insulin resistance
FINS	Fasting insulin
HIIT	High-intensity interval training
TLR4	Toll-like receptor 4
MyD88	Myeloid differentiation factor 88

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Author contributions
SJ, Xuan, and XW designed the study. SJ and Xuan drafted the manuscript. SJ and YT drew the figures and filled the table. XW, Jun and SJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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