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Liver Metaflammation in Diabetes is Mediated by JNK Signaling; Protective Effects of Combined Training

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Abstract
Objectives: Glucotoxicity-induced diabetes which aggravates chronic and progressive hyperglycemia of systemic glucose metabolism, leads to the activation of stress kinase -c-Jun N-terminal kinases (JNK) - for subsequent down regulatory catabolic responses and deterioration of β-cell function called liver metaflammation. Regarding the non-invasive property and anti-inflammatory effects of exercise training, we aimed to investigate the effects of 8 weeks of combined training (endurance and resistance), on the Gene expression levels of JNK, TNFα, Catalase, GPX, SOD as well as protein levels of *p*JNK and JNK in the liver tissue of type 2 diabetic male rats.
Methods: In this experimental study, 24 male Wistar rats (aged 8 weeks, 260±20 g) were randomly divided into 3 groups (n = 8/group), Normal Control (NC), Diabetic Control (DC), and Diabetes + Combined Training (DT). After the induction of diabetes, the training group implemented the combined training for 8 weeks. About 48 hours after the final training session, serum levels of glucose, insulin, and HOMA-IR along with gene expression and protein levels of *p*JNK, JNK, TNFα, Catalase, GPX, SOD were assessed through Elisa, RT-PCR, and Western blot techniques.
Results: Findings showed that 8 weeks of combined training significantly down regulated the serum levels of Insulin Resistance (IR) indexes (glucose, insulin, and HOMA-IR) and tissue levels of inflammatory indexes (*p*JNK, and TNFα); In contrast, combined training significantly increased tissue Enzymatic antioxidant activity (Catalase, GPX, SOD) compared to the diabetic group ($P< 0.05$).
Conclusions: Modality of Combined training (endurance + resistance) can reduce the risk factors associated with liver metaflammation of type 2 diabetic rats.

Keywords
Diabetes, Combined training, Metaflammation, JNK, Antioxidant

INTRODUCTION

The pancreas is the second largest heterocrine gland in the body that has pleiotropic function due to its contribution to the liver for metabolism regulation through the production of several metaboregulatory hormones (1); As an endocrine gland, the pancreas has a potential role of glucoregulatory effects through acute and chronic secretion of insulin and glucagon (2). From the physiological point of view, the metabolism of carbohydrates, particularly glucose, is crucial for

glycosylation processes and maintaining metabolic homeostasis (3). Disruptions in glucose regulation lead to the production of advanced glycation end-products (AGE) and receptors (RAGE) (4); As a response, the excessive production of insulin is recognized as a prominent symptom of the onset of the pathology condition (3,4); From a clinical perspective, hyperinsulinemia is considered a key factor in liver metaflammation. This can be caused by either an increase in pancreatic insulin secretion or a decrease in the liver's ability to clear insulin due to low hepatic uptake (5). Additionally, it can be associated with an increase in portosystemic/intrahepatic shunting. Excessive and prolonged insulin production, together with the harmful effects of high glucose levels, results in the depletion of pancreatic function (Known as ‘‘pancreas exhaustion’’) and the development of diabetes (5,6).

Insulin homeostasis perturbation prevents the liver from lipoproteins uptake and exports them to the circulation, resulting in fat accumulation (1,5,6). A recent discovery indicates that white adipose tissue functions as a gland, responsible for the secretion of pro/inflammatory cytokines and tumor necrosis factor-alpha (7). This process is believed to be the primary cause of lipotoxicity reactions, which are characterized by oxidative stress and subsequent inflammatory-induced necroptosis (8). The most prevalent FFAs are oleate (unsaturated) and palmitate (saturated), which are released into the bloodstream by excess visceral/subcutaneous fat deposits (9). Numerous mechanisms have been proposed to explain the adverse effects of lipid metabolites on tissues such as muscle, liver, and β-cells; These mechanisms include innate immune activation, endoplasmic reticulum (ER) stress, toxic fat esterification products (DAG and ceramides), mitochondrial dysfunction, which results in the chronic activation of c-Jun N-terminal kinases (JNK) (10). Ceramides also play a role in decreasing insulin gene transcription, regardless of JNK involvement (10,11). Conversely, the process of oligomerization and dissociation of Glutathione S-transferase pi (GSTpi) from JNK enables this kinase to phosphorylate (*p*JNK) and move from the nucleus to the cytoplasm, thereby initiating downregulatory catabolic reactions (12). The temporary activation of JNK in β-cells is responsible for promoting cell proliferation and differentiation, as well as initiating the appropriate cellular response to stress (13). However, if JNK remains elevated for an extended period of time, it inhibits insulin signaling and secretion, leading to β-cell failure (13, 14). This suggests that JNK plays a crucial role in determining the fate of cells by regulating metabolic activity in response to inflammation (13,14). A study by Lanuza-Masdeu et al. (2013) demonstrated that mice with the long-term elevation of JNK in β-cells exhibit glucose intolerance decreased insulin secretion, and disrupted insulin signaling within the β-cells (15). Research has demonstrated that in conditions of high levels of free fatty acids (FFA) and glucose, tumor necrosis factor-alpha (TNFα) is generated locally (16). These TNFα molecules can bind to and activate receptors on β-cells, leading to the activation of the JNK signaling pathway (17). In addition, lipid byproducts present in the cells, such as diacylglycerol (DAG), can stimulate protein kinase C (PKC), which in turn can activate NADPH oxidase (18). This enzyme generates superoxide molecules, often known as ROS, within the cytosol (18). The production of reactive oxygen species (ROS) can trigger the activation of c-Jun N-terminal kinase in a manner that is sensitive to changes in the redox state (19). Subsequently, superoxides and reactive nitrogen and oxygen species (RNOS) drive the major antioxidant defense system, which includes superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (20). This activation process is known as diapedesis (21). The β-cell is very susceptible to oxidative stress due to its limited concentration of antioxidant enzymes (15,17). Cytokines stimulate both hepatic and peripheral insulin resistance (IR) by a feed-forward process that inhibits the insulin receptor's ability to phosphorylate IRS1/2, leading to the propagation of IR throughout the body (18).

There are multiple techniques to regulate the inflammatory response caused by IR in the body; One of the non-invasive strategies is the implementation of regular physical exercise (22). While the impact of exercise training on immunological and oxidative stress responses is well-known in various body fluids, organs, and tissues, the effectiveness of different types of exercise training remains unclear in this field (23). Research has demonstrated that combined endurance and resistance training can reduce the presence of inflammation markers in the bloodstream of both healthy individuals and those with metabolic syndrome (24). This is achieved through increased glucose uptake, improved insulin sensitivity, and elevated levels of superoxide dismutase and catalase enzymes in the blood (24,25). Parastesh et al (2019) demonstrated that long-term implementation of combined training can stimulate anabolic responses by increasing testosterone levels and reducing insulin resistance through enhancing glucose uptake (24).

Despite all the conquered new knowledge and complex etiology of glucose metabolism, linking IR with oxidative stress and metaflammation indices, the puzzle is still incomplete and several pieces of information about down-regulating signals responsible for metabolic homeostasis seem to be missing. We hypothesize that diabete may affect liver glucose consumption pattern and oxidation homeostasis via JNK pathway, resulting in liver metaflammation symptom developmet; However, the effects of combined training on modulation of oxidative stress/inflammation have not been well documented; so we aimed to evaluate effects of combined training on serumic inflammation marker as well as prteomic levels of *p*JNK/JNK as well as anti-oxidant enzymes changes in the liver tissue of dibetic rats.

MATERIALS AND METHODS

Ethical approval

Animal care and ethical principles were based on the guide for the care and use of laboratory animals (26) approved by the Ethics Committee of AJA University of Medical Sciences [IR.AJAUMS.REC.1401.034].

Animal model and experimental design

In this experimental study, 24 male Wistar rats (8 weeks old, weighing 260±20 grams) were obtained in the Center of Razi Animal Institute, Karaj Medical University. The rats were 8 weeks old and had an average weight of 260±20 grams. Animals were provided with unlimited access to food and water before and during the experiment. Upon arrival at our laboratory animal experiment site, the rats were given one week to adjust to the environment. The conditions during this period were maintained at a temperature of 24 ± 1 °C, with a humidity level of 45% to 55%, and a 12:12 dark/light cycle. The rats had unrestricted access to water and were provided with standard chow, which consisted of 54% mixed carbohydrate, 19% protein, and 3% lipid. Following this time frame, the rats were allocated into three groups using a random selection process: Normal Control (NC), Diabetic Control (DC), and Diabetic + Combined Training (DT).

Induction of Type 2 Diabetes

The rats in the D and D+COT groups were given a single dosage of nicotinamide (made by Qualchems Company) and STZ solution (produced by Sigma Aldrich Company) diluted in sodium citrate buffer (0.1 M, pH = 4.5). The injection was administered intraperitoneally at a dose of 60 mg/kg of the animals' weights. The fasting blood glucose (FBG) level was assessed using the Glucometer (AccuChek Active Performa, Roche, Germany) 3 days (72 hours) following the administration of STZ to verify the induction of diabetes. Rats with blood glucose levels ranging from 220 to 250 mg/dl were classified as having overt diabetes. Day 4 of the experiment, which occurred 72 hours after, was designated as the day of confirmation of diabetes induction (27).

Training protocol and load measurements

After the induction of diabetes and a week of adaptation to treadmill running (10 minutes at a speed of 5-10 m/min) and the resistance ladder (10 repetitions with weights of 5-30% of the body weight of mice), the combined training protocol performed alternative sessions of endurance training followed by resistance training 5 days a week and for 8 weeks. Endurance training was performed running at a velocity of 20 meters/min (~55-65% of Vo2max) and gradually increased to 30 meters/min for a total time of 60 minutes in the 8th week. Each training session further consisted of a 10-minute warm-up and cool-down (the speed; was 10 m/min at 0º incline). For strength training, rats ascended a 1-meter length ladder with a 90º slope in each session for 10 repetitions with a weight of 50%, 75%, 90%, and 100% of their maximal repetition, which was attached to their tail. The amount of one maximum repetition was measured at the end of each session by adding weights of 30 grams until fatigue (24).

Tissue extraction and blood collection

Following an 8-week treatment period, the rats that had been fasting for 12 hours were given an intraperitoneal injection of a mixture of ketamine and xylazine (at doses of 80 and 12 mg/kg, respectively) 48 hours after their final exercise session. Cardiac blood samples were extracted and centrifuged at a speed of 2500 rate per minute for a duration of 15 minutes at a temperature of 4 degrees Celsius. The resulting plasma was then maintained at a temperature of -20 degrees Celsius until glucose and insulin assays were conducted. The liver tissue was removed and promptly frozen using liquid nitrogen. It was then maintained at a temperature of -80 ◦C until it was ready to be processed for western blot and biochemical analysis.

RT-PCR quantification

In summary, the liver tissue was subjected to total RNA isolation using the RNeasy Cybergreen kit (Qiagen Inc., Valencia, CA) or Trizol reagent (Ampliqon, Denmark). The Tissue Homogenizer system (Kavoshmehr, IR) was employed for tissue homogenization. A High-Capacity cDNA Reverse Transcription Kit (Yekta Tajhiz Azma, IR) was used to create complementary DNA from 2 µg of total RNA. To assess the amounts of messenger RNA (mRNA) for 5 genes associated with lipolysis (TNFα, SOD, CAT, and GPx), we utilize quantitative real-time polymerase chain reaction (qPCR). The reactions were conducted using 40 amplification cycles: 10 minutes at 25 ◦C for denaturation, 120 minutes at 37 ◦C for annealing, and 5 seconds at 85◦C for extension. The RT-PCR assay was performed on a Corbette thermal cycler (Corbette, Italy) using Taqman GE Master Mix for detection. The B-actin gene was amplified as a housekeeping gene. The fold change in gene expression was determined using the comparative 2–ΔΔCt technique, as previously suggested (28).

Table 1 RT-PCR forward and reverse primer sequences

Gene	NCBI accession number	Reverse and forward primer	Amplicon Size
β-actin	NM_031144.3	Forward: GTGTGACGTTGACATCCGTAAAGAC	119
		Reverse: ACCGTGCCTTCAGTGTGCTTC	
SOD	NM_017051.2	Forward: CCCTGACCTGCCTTACGAC	140
		Reverse: CGTGGTACTTCTCCTCGGTG	
CAT	NM_012520.2	Forward: ATCAGGTTACTTTCTTGTTCAGCG	147
		Reverse: TGATGCCCTGGTCAGTCTTG	
GPX	NM_030826.4	Forward: AGTGCGAGGTGAATGGTGAGA	146
		Reverse: CCAGGAAATGTCGTTGCG	
TNFα	NM_012675.3	Forward: CCGAGATGTGGAACTGGCA	133
		Reverse: CAGTAGACAGAAGAGCGTGGTG	

Western Blotting

The protein content was quantified using the Bradford method. In summary, approximately 10 mg of liver tissue was pulverized using stainless steel balls for 1 minute in a Mikro-Dismembrator S (Sartorius, Goettingen, Germany). The resulting tissue was immediately homogenized in a lysis buffer consisting of 500µL Tris-HCL (pH 8), 0.08 gr NaCl, 0.003gr EDTA, 0.025gr Sodium Deoxycholate, 0.01gr SDS, 1 tablet of protease inhibitor cocktail, and 10 µl of NP40 (1% Triton). Next, the lysate underwent centrifugation at a rate of 12,000 times the acceleration due to gravity at a temperature of 4 °C for 15 minutes. The liquid portion above the sediment, known as the supernatant, was then poured off. The supernatant obtained was mixed with electrophoresis loading buffer, which consisted of 0.6 ml Tris-HCl (pH 6.8), 0.2 grams of SDS, 2.5 milligrams of glycerol, 0.5 milligrams of β-mercaptoethanol, and 0.01 milligrams of bromophenol blue. Once the linearity of protein quantities in each sample was verified, an equivalent quantity of protein (12 µg) was loaded onto SDS-PAGE and subsequently transferred to Immun-Blot polyvinylidene fluoride (PVDF) membranes. The variability of the experiments and the efficiency of loading and transfer were precisely quantified using a total protein staining approach including Reactive Brown. The membranes were blocked for 1 hour in a solution containing 4% bovine serum albumin. They were then rinsed in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and exposed to the primary antibodies. The primary antibodies used were polyclonal mouse JNK (SC-7345) and polyclonal mouse *p*JNK (SC-6254), both diluted at a ratio of 1:300 in TBST. The housekeeping gene employed was β-Actin, diluted at a ratio of 1:2000 using TBST (sc-47778, Santa Cruz). After incubating the membranes with primary antibodies, they were then incubated with an HRP-conjugated anti-mouse antibody. The antibody was diluted in a 5% Blotto blocking buffer at a ratio of 1:5000 to 1:20000. Chemiluminescent visualization was performed using Clarity™ Western ECL Substrate (ECL, Amersham, UK) with enhanced chemiluminescence detection kit. The visualization was done before the signal reached saturation on an X-ray film (Fuji, Japan). Finally, the band densitometric data were quantified using densitometric analysis software Image J 1.6.0. As the loading was uniform across all membranes, no additional adjustments were made. Fig. 2 shows representative immunoblots.

Eliza assessments

Enzyme-linked immuno-absorbent assay (ELISA) method used to determine serum glucose (Pars Azmon kit, Karaj, Tehran), and insulin (ZellBio, Germany, Cat. No : ZB-10707C-R9648) levels.

Statistical Analysis

All data were presented as means ± SEM. Groups comparisons were achieved using one-way ANOVA, followed by the Tuckey post-hoc test. Statistical significance was considered at $P<0.05$. The statistical analysis was performed using SPSS statistical software (Version 22).

RESULTS

The results of diabetes induction (via *nicotinamide* and *STZ* injection) and combined exercise on body composition, lipid profile (LDL, HDL, total cholesterol, and triglyceride), glucose, insulin, and HOMA-IR index, along with inflammatory index and Serum oxidative stress as well as protein levels of JNK and pJNK peptides in the liver tissue of the rats are presented in this section.

Body weight changes

The results of Diabetes induction followed by 8 weeks of combined training show a significant difference in final weights ($P=0.26$), Weight changes ($P=0.033$), Inguinal fat pads ($P=0.001$), Subcutaneous fat pad ($P=0.012$), Inguinal to final weight ($P=0.001$), Subcutaneous to final weight ($P=0.001$) of study subjects (Table 2). The results of Tuckey's post hoc test showed that the induction of diabetes increased the visceral and subcutaneous fat weights in the DC group compared to the NC group, and implementation of the combined training modulated the subcutaneous and visceral fat pads (Table 2). Among other findings of the current research, we can mention the decrease of fat pads to the NC level under the effects of exercise intervention (Table 2).

Serum Lipid Profile (LDL, HDL, total cholesterol and triglyceride)

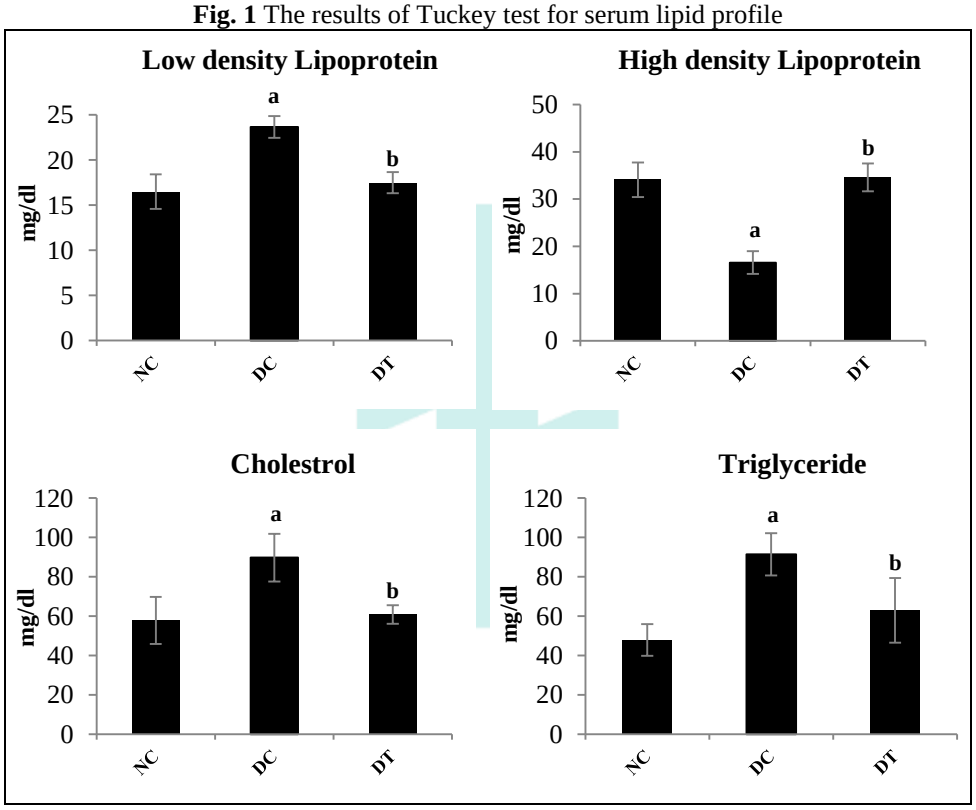
Figure 1 shows the changes in the lipid profile of rats during 8 weeks of diabetes intervention and combined training among the study groups. The results of the ANOVA test (one-way) show that there is a significant difference in low-density lipoprotein ($p=0.0007$), high-density lipoprotein ($p=0.0001$), total cholesterol ($p=0.0044$) and triglyceride ($p=0.0091$) among the study groups. On the other hand, the results of the Tuckey post-hoc test showed that the induction of diabetes led to an increase in the serum levels of lipid profile risk factors (LDL, Cholesterol, Triglyceride) and a decrease in HDL (Figure 1). Also, the combined exercise protocol had an equal ($p<0.001$) modulating effect on serum LDL, Cholesterol, Triglyceride, and HDL compared to the NC group (Fig. 1).

Table 2 Weight ratios (of different fat tissues) of rats

	NC (N=8)	DC (N=8)	DT (N=8)	P_{Value}
Final weight (g)	405.2 ± 18.38	356.8 ± 19.30[a]	320 ± 24.51[a,b]	0.26
Weight changes (g)	135.68 ± 24.50	109.25 ± 25.73	62.7 ± 32.68	0.033 *
Inguinal fat pads (g)	2.35 ± 0.33	5.92 ± 0.64	2.09 ± 0.23	0.001 *
Subcutaneous fat pad (g)	2.45 ± 0.35	4.52 ± 0.57	2.14 ± 0.1	0.012 *
Inguinal to final weight (%)	0.82 ± 0.1	1.69 ± 0.2	0.87 ± 0.1	0.001 *
Subcutaneous to final weight (%)	0.68 ± 0.1	1.18 ± 0.1	0.69	0.001 *

Data expressed as Mean ± SD; * significant group difference at $P< 0.05$ and ** at $P< 0.01$ according to ANOVA. NC: Normal Control; DC: Diabetic Control; DT: Diabete + combined training. [a] versus the NC group; [b] versus the DT group according to Tuckey test ($P<0.05$)

Fig. 1 The results of Tuckey test for serum lipid profile

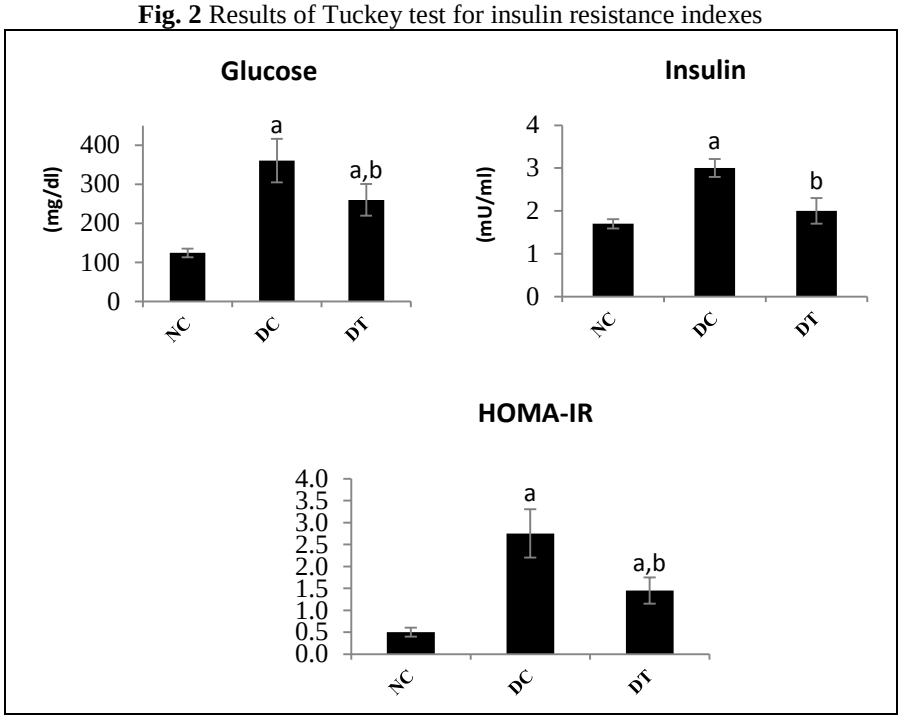


Data expressed as Mean ± SD; NC: Normal Control; DC: Diabetic Control; DT: Diabete + combined training. [a] versus the NC group; [b] versus the DT group according to Tuckey test (P<0.05).

Serum Glucose, Insulin and HOMA-IR index

HOMA-IR index, serum glucose and insulin concentration in DC group was higher than NC group (relatively P=0.002, P=0.002 and P=0.001) by the means of STZ injection has induced insulin resistance (Fig.2). Combined training was able to reduce HOMA-IR index (P=0.039) through down-regulation of insulin (P=0.007) but not glucose (P=0.231) in comparison to DC group (Fig. 2).

Fig. 2 Results of Tuckey test for insulin resistance indexes

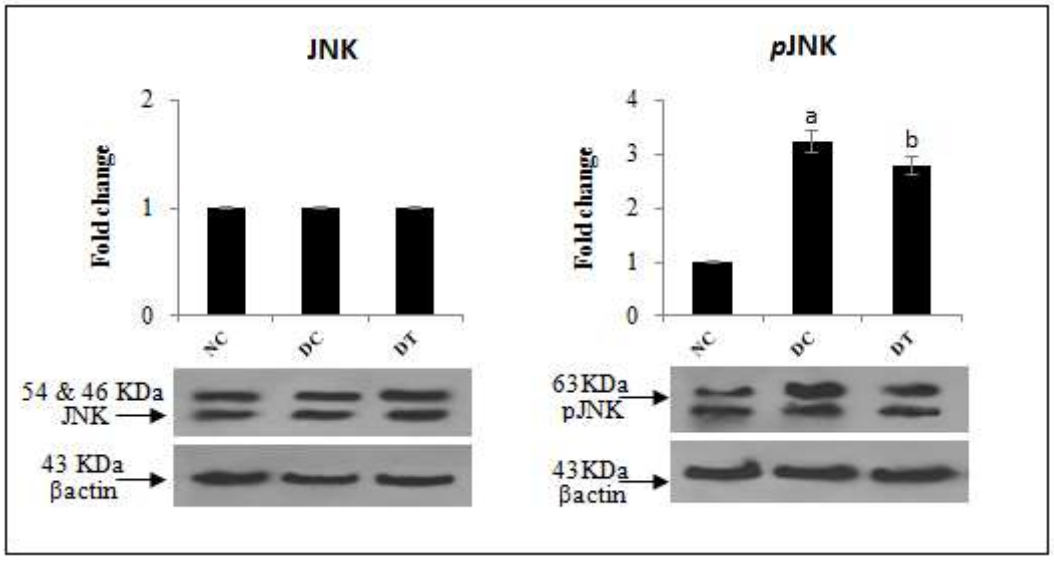


Data expressed as Mean ± SD; NC: Normal Control; DC: Diabetic Control; DT: Diabete + combined training. [a] versus the NC group; [b] versus the DT group according to Tuckey test (P<0.05)

JNK and pJNK Protein expression levels

Protein densitometry analysis of liver tissue showed that diabetes induction increased the protein levels of *p*JNK (P=0.017) with no effects on JNK (P<0.001) levels in the DC group compared to the NC group. Combined training was able to modulate pJNK (p=0.019) in comparison to the DC group (Figure 3).

Fig. 3 The effects of T2M and exercise on protein expression levels of JNK and pJNK

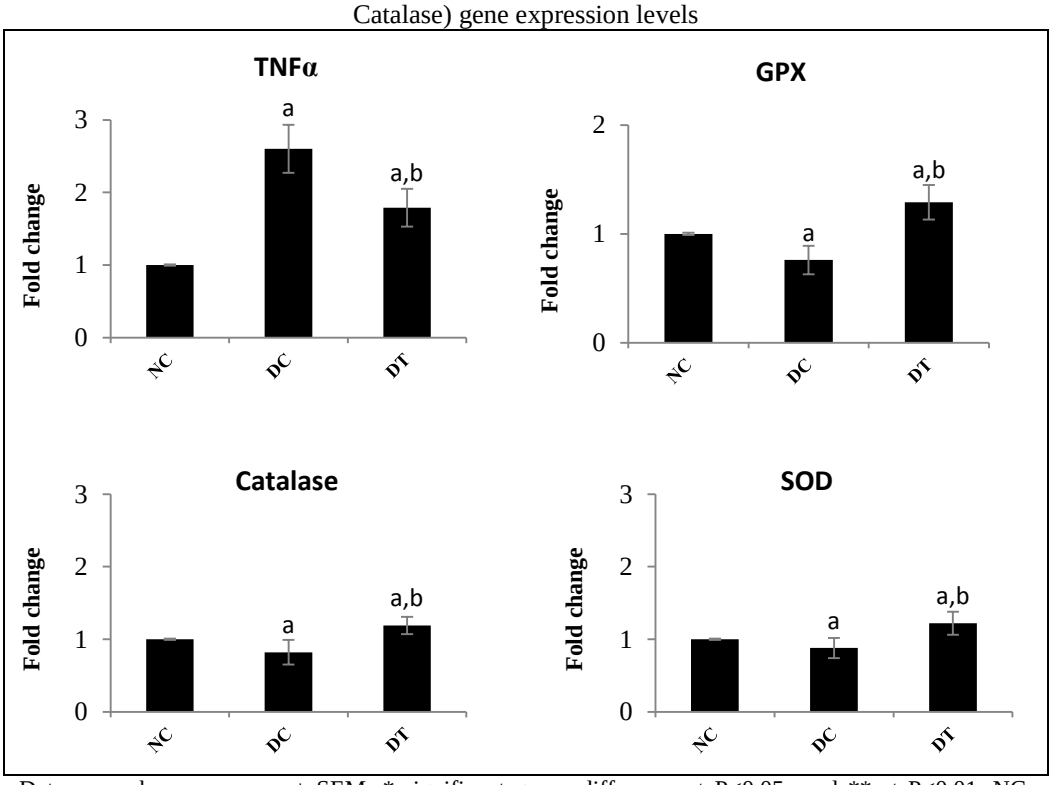


Data were shown as mean ± SEM; * significant group difference at P<0.05 and ** at P<0.01; NC: control; DC: Diabetic; DT: Diabetic + Combined Training. a versus the NC group; b versus the DT group

Gene expression levels of TNFα, Catalase, GPX and SOD in Liver tissue

Concerning serum inflammatory and anti-oxidant markers, our analysis revealed that diabetes induction is related to increased liver tissue gene expression levels of the inflammatory index, TNFα (*P* = 0.003), and decreased gene expression levels of anti-oxidant markers, GPX (*P* = 0.037), SOD (*P* = 0.033) and catalase (*P* =0.0204); Combined training was able to modulate these detrimental effects of diabete through down-regulation of TNFα(*P* =0.0204) and ameliorated GPX (*P* = 0.012), SOD (*P* = 0.047) and catalase (*P* =0.011) in comparison to DC group (Fig. 4).

Fig. 4 The results of Tuckey test on inflammatory (TNFα) and antioxidant (GPX, SOD and Catalase) gene expression levels



Data were shown as mean ± SEM; * significant group difference at P<0.05 and ** at P<0.01. NC: control; DC: Diabetic; DT: Diabetic + Combined Training. a versus the NC group; b versus the DT group

DISCUSSION

The novel findings of this study emphasized that regular combined training, even in the presence of hyperglycemia, reversed STZ-induced metaflammation symptom development in part by reducing adipocyte size, elevated anti-oxidant activity, and ultimately HOMA-IR attenuation. All these changes lead to the improvement of liver function and attenuation of liver pathology.

Effects of Diabete and combined training treatment on Body weight, fat pads and lipid profile

It is well known that diabetes consequences impact body weight through changes in lipid profile (29). Despite the results of other studies, the animals induced diabetes in our study, achieve greater weight gain compared to the basal levels, both absolute and relative. This could be attributed to the diabetes induction model; Nicotinamide has shown protective effects against cytotoxic effects of STZ that avoid weight loss (30) in the rats of the present study; parallelly, diabetes induction through only STZ shown to weight losses (31); but STZ plus NA injection has been shown to increase weights of the rats (32). Also, STZ injection dose counts as a dominant factor in weight alterations; in this field, Bauer et al (2023) examined dose-dependant (25 and 55 mg/kg) manners of STZ injection on albino rats weight variations and observed both weight gain and weight loss respectively in low and high dose of STZ injection (33). This could be the probable mechanism of weight gain that occurred in the present study due to the high dose of STZ injection. The cooccurrence of obesogenic factors, such as higher amounts of subcutaneous and inguinal fat pads, as well as elevated levels of serum triglycerides, total cholesterol, VLDL, and LDL-c, combined with a relatively lower weight gain compared to the NC group, provides evidence for the classification of an atrophy status caused by diabetes induction (34), which was also observed in our study. However, it is worth noting that many cells in various organs, including the liver, possess the potential to generate fat through a process called de novo pathway in situations where there is an excess of energy, such as hyperglycemia, the liver converts glucose into glycogen (35). Prolonged exposure to hyperglycemia can lead to the saturation of glycogen stores and the conversion of excess glucose into free fatty acids (FFA) via the de novo pathway (35). The fatty acids can be subsequently synthesized into triglycerides and transported out of circulation (35). Liver diabetic dyslipidemia occurs due to an imbalance in the transportation of lipids to the liver and their subsequent uptake, synthesis, oxidation, and secretion (36). This imbalance leads to dyslipidemia and the death of β-cells by apoptosis (36). An elevated concentration of triglyceride-rich lipoproteins causes an increase in the breakdown of HDL (resulting in low HDL-C) and a change in the LDL phenotype towards low-density lipoprotein (LDL), which are more likely to cause atherosclerosis compared to "normal" LDL (37) that was observed in the present study. The study demonstrates that long-term exposure to high blood sugar levels and abnormal lipid levels leads to an increase in HOMA-IR, which is a marker of insulin resistance (38). This may be due to various mechanisms, including the role of HDL in regulating glucose metabolism (38). Higher levels of HDL-C are associated with lower blood sugar levels (39). Additionally, HDL promotes the transport of cholesterol out of cells, and the changes in lipid levels within cells are thought to reduce inflammation (39). Recent research has demonstrated that fat accumulation can alter the composition of lipid membranes and lead to an increase in intestinal

permeability (40). This, in turn, is linked to higher levels of systemic inflammation and a heightened risk of developing Type 2 Diabetes (41). Skeletal muscle acts as the primary storage site for glycogen accounts for over 30% of energy expenditure (42) and counts as the main peripheral tissue that is primarily responsible for glucolipid metabolism under the condition of IR (42). Our study found that combined training effectively prevented an increase in body weight by reducing the size of fat pads. This was accompanied by a decrease in blood levels of LDL-c, total cholesterol, triglyceride, and VLDL, as well as an increase in serum HDL-c. The implementation of combined training may have reduced the dyslipidemia caused by hyperglycemia. This could be due to increased activity of antioxidant enzymes, enhanced leptin activity and energy expenditure, and the subsequent activation of highly sensitive lipase and β subunit of AMP-activated protein kinase (AMPK) (43). These factors lead to increased lipid participation in the metabolic process and improvement in body composition (43). Endothelial dysfunction is considered a significant factor in insulin resistance (44). This dysfunction is characterized by a decrease in the secretion of nitric oxide from endothelial cells (44). Insulin, through its nitric oxide-mediated function, promotes glucose uptake in skeletal muscle (45). However, in cases of insulin resistance, there is an increase in inducible nitric oxide synthase (iNOS) in the bloodstream, which leads to the downregulation of insulin receptors, including IRS1 and IRS2 (44). Consequently, this prevents insulin from binding to its receptor (44). Prior research has indicated a clear correlation between elevated levels of circulating iNOS and elevated fat storage (46). The implementation of the combined training protocol in this study appears to have activated the enzyme eNOS (Endothelial nitric oxide synthase) through shear stress, resulting in improved endothelial function and reduced levels of vascular inflammation factors, such as iNOS (inducible nitric oxide synthase). These factors play a role in modulating insulin resistance and subsequent inflammatory status (44, 46), which aligns with the findings of our study.

Finally, it may be inferred that the combined training strategy enhanced insulin sensitivity by promoting the absorption of glucose by active muscle during exercise through the up-regulation of glucose transporter-4 (47). The observed glucoregulatory outcome was expected as a consequence of the combined training sessions, as this training protocol heavily relies on the glycolysis system for energy generation (47). Parastesh et al (2018) observed an enhanced insulin resistance status in diabetic rats due to their adaptation to mixed training, which included both endurance and resistance exercises (24).

Effects of Diabete and combined training on oxidative stress and inflammatory markers

Scientific evidence has demonstrated that diabetes causes an increase in blood sugar levels, known as hyperglycemia (41,47). Additionally, prolonged exposure of beta cells in the pancreas to high levels of fatty acyl-CoA hinders the release of insulin by activating the Randle cycle (48). In addition, elevated levels of fatty acyl-CoA in the beta cells also promote the production of ceramide, which enhances the activity of inducible nitric oxide synthase (iNOS) (11). The resulting elevation in nitric oxide levels enhances the production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα), which hinder the function of beta cells and cause the death of beta cells (49). The initial significant connection established between inflammation and obesity was the identification of the pro-inflammatory cytokine TNFα (16,17). Subsequent research has discovered that TNFα has the ability to disrupt insulin signaling, leading to insulin resistance (16,17). Tumor necrosis factor α (TNFα), a cytokine that is commonly raised during obesity and type 2 diabetes (T2D), activates the TNF receptor, which in turn activates TAK1 (10). TAK1 then selectively activates MKK7, and in conjunction with MKK4, leads to the activation of JNK as a stress kinase (10). Furthermore, the presence of saturated free fatty acids (FFA), such as palmitate, can induce endoplasmic reticulum (ER) stress, which in turn triggers the activation of the JNK catabolic pathways (50). The current research focuses on investigating the role of inflammatory kinases, including JNK, as a significant contributor to both insulin resistance and ß-cell dysfunction. Short-term JNK activity may lead to cell growth, but sustained activation could induce cell demise (13). Macrophages release pro-inflammatory cytokines, which contribute to a feed-forward cycle by attracting more macrophages and spreading inflammation to other insulin-sensitive tissues through the bloodstream (51). Within cells, fat metabolites, such as diacylglycerol (DAG), have the ability to stimulate protein kinase C (PKC), which in turn can activate NADPH oxidase (52). This enzyme generates superoxide molecules, also known as reactive oxygen species (ROS), within the cytosol (20). During the state of inflammation, the production of superoxides and reactive nitrogen and oxygen species (RNOS) triggers the activation of the main antioxidant defense system, which includes superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (20). Exercise training has been found to enhance the enzymatic responses of antioxidants (53). Specifically, engaging in high-intensity/load movements during combined exercise can cause microfractures in muscle fibers (54). This, in turn, triggers the body's first line of defense against oxidative stress in bodily fluids and organs during the recovery phase (55). This could be the probable modulatory mechanism of adaptation to 8 weeks of combined training that resulted in the down-regulation of serum TNFα and liver JNK in the present study. Furthermore, our previous research observed that aerobic exercise improved lipid metabolism, decreased lipid droplet accumulation, and increased antioxidant activity in the testes tissue of insulin-resistant rat models (55). In a study conducted by Cui et al (2022), the researchers examined the long-term effects of swimming training on the inflammatory responses of rats with non-alcoholic fatty liver disease (NAFLD). They found that exercise training had a modulating effect on inflammatory responses, specifically the JNK pathway, and reversed the process of cell death by reducing lipid levels in the rats' bloodstream (56).

The implementation of combined training has been shown to decrease the phosphorylation of JNK in the liver through various mechanisms. It is hypothesized that this effect is likely due to the stimulation of shear pressure, which

leads to vasodilation, This vasodilation has been demonstrated to modulate insulin resistance and reduce the levels of inflammatory markers (57). Another potential mechanism for the effects of a combined training regimen could be the activation of mitochondrial biogenesis. Research has demonstrated that TNFα can reduce PPAR isoforms, which in turn restricts the expansion of adipose tissue and promotes the storage of fat in other areas of the body (58). FFA may stimulate the JNK and NFκB pro-inflammatory pathways, leading to heightened production of pro-inflammatory cytokines, including TNFα and IL-6 (10). In addition, it is believed that the combination of resistance training and endurance activity can impose a significant load on the body, leading to anabolic adaptation (59). This is routed from different energy systems recruitment and different types of muscle fibers for energy production during the activity (59). As a result, there is an increase in mitochondrial content and subsequent enhancement of mitochondrial biogenesis, which is regulated by PPAR isoforms (58). Due to its pleiotropic properties, the combined exercise protocol has been extensively studied by various researchers (59). De Matos et al () investigated the acute effects of Combined training in an obese patient with IR; the results showed that Combined training causes a drop in serum JNK levels by a possible mechanism of increased HSP70 and HSP72 levels that directly bind to the JNK and abrogation of this peptide from Phosphorylation and translocation to the nucleus and prevented catabolic responses (60).

The advantage of this study is the simultaneous assessment of serum and proteomic markers of oxidative stress and inflammation, allowing us to make more confident statements regarding the modulation of the inflammatory condition and improving the Liver function. Among the limitations of the present study, we can point out the lack of post-translational peptide fluctuations assessment in the liver due to diabetes induction and implementation of combined exercise, which could have increased the generalizability of the results of the study. This suggests that future research should also examine the post-translational factors in liver tissue to obtain a more accurate conclusion of the mechanism of chronic exercise activity on the modulation of inflammatory indices.

CONCLUSION

The current study provides novel and important insights into the management of IR to MetS progression and suggests that adaptation to non-invasive combined training treatments might avoid perturbation of redox balance in body fluids and tissues and be considered as an alternative treatment for the diabetic population that suffers from metaflammatory side-effects.

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AUTHORS' CONTRIBUTION

All authors contribute to the study design. Seyedeh Fatemeh Zarrini collected the data. Nader Hamedchaman and Mohammadreza Esmaelzadeh Toloee revised the final version of the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL CONSIDERATIONS

This study followed the ethical standards and was approved by the Ethics Committee of the AJA University of Medical Sciences [IR.AJAUMS.REC.1401.034].

DATA AVAILABILITY STATEMENT

The dataset presented in the study is available on request from the corresponding author during submission or after its publication.

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