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Continuing Education for Nutrition Professionals

Glucose Control Associations with Muscle and Resveratrol

Positive association between musclin and insulin resistance in obesity: evidence of a human study and an animal experiment

Abstract

Background: Musclin is a novel skeletal muscle-derived secretory factor considered to be a potent regulator of the glucose metabolism and therefore may contribute to the pathogenesis of obesity and insulin resistance (IR).

Methods: To test this hypothesis, we examined the plasma musclin levels in overweight/obese subjects and lean controls. Rats on a high fat diet (HFD) were used as the annimal model of obesity. Radioimmunoassay and western blot were used to determine musclin levels in plasma and skeletal muscle.

Results: According to radioimmunoassays,the overweight/obese subjects exhibited elevated musclin plasma levels compared with the lean controls (89.49 ± 19.00 ng/L vs 80.39 ± 16.35 ng/L, $P < 0.01$). The musclin levels were positively correlated with triglyceride, fasting plasma glucose, and homeostasis model assessment of IR levels. These observations were confirmed with a high-fat diet(HFD) rat model. HFD rats also exhibited increased musclin immunoreactivity in plasma ($P < 0.01$) and in skeletal muscle ($P < 0.05$), as well as increased musclin mRNA levels in skeletal muscle ($P < 0.01$). Musclin incubation significantly inhibited muscles ^{3}H-2-DG uptake in the normal diet(ND) group ($P < 0.01$). The protein expression of glucose transporter type 4 was significantly down regulated by 30% ($P < 0.05$) in the ND group after soleusmuscle was incubated with musclin compared with the control. Musclin incubation also increased the protein levels of glucose-regulated protein (GRP)78 and GRP94 by 146.8 and 54% (both $P < 0.05$), respectively, in ND rats.

Conclusions: Our data support the hypothesis that musclin has a strong relationship with obesity-associated IR by impairing the glucose metabolism and, at least in part, through causing endoplasmic reticulum stress.

Keywords: Musclin, Obesity, Insulin resistance, Skeletal muscle, Endoplasmic reticulum stess

Background

Individuals with obesity are at a higher risk for obesity-associated medical conditions, such as metabolic syndrome, and insulin resistance (IR), which can develop into type 2 diabetes mellitus (T2DM) [1]. Although the precise mechanism linking obesity to IR and T2DM are unknown, extensive evidence suggests that impaired glucose disposal in skeletal muscle plays a crucial role in development of obesity-associated disease [2]. Recently, it has been shown that skeletal muscle is an endocrine organ that can regulate energy metabolism homeostasis by releasing a variety of bioactive factors [3]. Pedersen et al. reported that cytokines and peptides released by muscle fibers exert either paracrine or endocrine effects and could be classified as “myokines” [4]. To date, a number of studies have shown that myokines, such as interleukin-6 (IL-6), interleukin-15 (IL-15) and brain-derived neurotrophic factor(BDNF), may be potential regulators of many physiological states and metabolic diseases, such as obesity and IR [5–8]. However, the connections between obesity, myokine secretion, and metabolic dysfunction remain to be elucidated. Among myokines, musclin is a newly

discovered, 130-amino acid peptide that was first reported by Nishizawa et al. Musclin has been found to be almost exclusively expressed in skeletal muscles [9]. Musclin is a pleiotropic myokine that is involved in the regulation of energy homeostasis. Nishizawa et al. reported that musclin mRNA expression was augmented in the skeletal muscle of obese mice [9]. In vitro studies have demonstrated that musclin significantly inhibited insulin-stimulated 2-deoxy-D-[1-3H]-glucose (2-DG) uptake and glycogen synthesis [9]. Furthermore, Liu et al. reported that pre incubating muscles with musclin reduced protein kinase B activation in the insulin-signaling cascade [10]. Although musclin has been shown to be a novel, endogenous obesity-related factor in animal models, the mechanism of its bioactivity and its expression in humans remain largely unknown.

In this study, we investigated the circulating levels of musclin and the clinical parameters associated with musclin levels in subjects from a human cohort with or without obesity. Furthermore, a high-fat diet (HFD) rat obesity model was used to test the hypothesis that musclin plays a role in obesity-associated IR in skeletal muscle.

Methods

Animals and reagents

Male Sprague–Dawley (SD) rats (155 ± 5 g) were provided by the Animal Department, Peking University Health Science Center. All animal care and experimental protocols complied with the Animal Management Rules of the People's Republic of China (Ministry of Health, P.R. China, document no. 55, 2001) and the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Care Committee of Health Science Center, Peking University. Musclin peptide was synthesized by Phoenix Pharmaceuticals (Belmont, CA, USA). [^{3}H] Deoxy-glucose was obtained from PerkinElmer (Boston, MA, USA), and insulin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against GRP78, GRP94 were from Abcam (Cambridge, UK). Antibodies against musclin and β-actin and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against GLUT-4 was from Wuhan BOSTER Bioengineering (Wuhan, Hubei, China).

Preparation of the animal model

Male SD rats (155 ± 5 g) were randomly divided into the 2 following groups (n = 8 each): the normal diet (ND) group, comprising 14,610 kJ/kg and energy contents (%) for carbohydrates, fat, and protein of 66.50, 10.21, and 23.29, respectively; and the HFD group, comprising 19,315 kJ/kg, 200 g fat/kg (170 g of lard +30 g of corn oil to provide essential fatty acids) and 1% cholesterol by weight plus normal drinking water. The HFD was formulated to provide 40% of the total energy from fat by replacing carbohydrate energy with lard and corn oil energy. The HFD had the same amounts of vitamins and minerals per kilojoule as the ND [11]. After 2 days of further treatment, the rats were anesthetized with urethane (1 g/kg, intraperitoneally) at the end of a dark cycle and overnight fasting and were then euthanized. Blood was collected in a heparinized syringe from the abdominal aorta and mixed with 1 mg/mL EDTA-2Na and 500 KIU/mL aprotinin or heparin. Plasma and serum were obtained by centrifugation at 3000 rpm for 10 min at 4 °C and stored at −70 °C. White gastrocnemius muscles were collected and weighed. All tissue samples were frozen in liquid nitrogen.

Subjects

The study was performed in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University, Harbin, China. Written informed consent was obtained from all participants prior to participation. We selected 117 consecutive subjects from the general population who had undergone medical check-ups at the Outpatient Department of the Second Affiliated Hospital of Harbin Medical University. Exclusion factors were diseases affecting the metabolic state or being unsuitable for participation in this study. All subjects completed a self-administered questionnaire to provide data on age, smoking history, alcohol consumption, history of T2DM and medications. All subjects underwent a physical examination, including measurements of height and weight (while wearing light clothes and no shoes), body mass index (BMI, kg/m^2), and blood pressure. Waist circumferences were calculated midway between the iliac crest and rib cage and were rounded to the nearest 0.1 cm;hip circumference was measured at the point of the maximum extension of the buttocks. Waist-to-hip ratios were calculated by dividing the waist circumference (cm) by the hip circumference (cm). According to the recommendations of the Working Group on Obesity in China, subjects with BMI between 18.5 and 24.0 kg/m^2 were considered normal, while those with BMIs between 24.0 and 28.0 kg/m^2 or over 28.0 were defined as overweight or obese [12].

Radioimmunoassays of musclin levels in muscle and plasma

Musclin levels were measured using a specific, commercially available radioimmunoassay kit (Beijing Sino-UK Institute of Biological Technology, Beijing, China). Gastrocnemius muscles were immediately acidified by the addition of 1.0 mol/L acetic acid and were then heated at

100 °C for 10 min to inactivate proteases. Tissue homogenates were prepared using a Polytron homogenizer, centrifuged at 17,000×g for 20 min, and then the supernatants collected. The plasma samples were pre-treated with aprotinin (500 KIU/mL). The assay sensitivity provided by the kit manufacturer was 1.25 pg/mL, and the standard curve range was from 0 to 400 pg/mL. The assays showed a good degree of parallelism. The intra- and interassay coefficients of variation were validated in the present study and were 7.1 and 10%, respectively. There was no cross-reactivity with rat IL-15, insulin-like growth factor-1, platelet-derived growth factor, fibroblast growth factor, transforming growth factor-β or hepatocyte growth factor.

Glucose uptake

After fasting overnight, rats were anesthetized using urethane (1 g/kg, intraperitoneally) and euthanized; the soleus muscles were obtained by dissection (25-30 mg) and preincubated in 12-well plates at their resting length. The soleus muscles were then incubated in 2 mL of Krebs-Henseleit buffer (KHB) containing 40 mmol/L mannitol, 0.1% bovine serum albumin (BSA) and 8 mmol/L glucose with or without 2 mIU/mL insulin at 37 °C for 2 h. The muscles were then transferred to 12-well plates in 2 mL of KHB containing 40 mmol/L mannitol, 0.1% BSA, 1.5 μCi/ml [^{3}H]-2-deoxy-D-glucose and insulin at the same concentration as was used during the preceding incubation. The plates were placed in a water bath at 37 °C under continuous shaking (60 beats/min) and bubbling of 95% O_2 and 5% CO_2. After 30 min, the muscles were placed in scintillation vials containing 100 μL of formic acid and 100 μL of 30% hydrogen peroxide and were counted in a Packard liquid scintillation counter with channels preset for simultaneous ^{3}H [13, 14].

Muscle preparation and incubation

After an overnight food restriction, the rats were anesthetized by urethane (1 g/kg, intraperitoneally) and euthanized. The soleus muscles were dissected (25-30 mg) with a bistoury and were preincubated for 30 min in 12-well plates at their resting length in KHB with 2% BSA. After preincubation, muscles were incubated in KHB with or without 1.5×10^{-7} mol/L musclin for 3 h, as previously described. The plates were placed in a water bath at 37.8 °C under continuous shaking (60 beats/min) and bubbling with 95% O_2 and 5% CO_2. After co-incubation for 3 h, the tissues were rapidly blotted on ice-cold filter paper and stored at −80 °C until the Western blot analysis.

Real-time PCR analysis

Total RNA from the gastrocnemius muscles was isolated and reverse transcribed using a reverse transcription system (Promega, Madison, WI, USA). In total, 20 μL of the reaction mixture underwent real-time PCR. The amount of PCR product formed in each cycle was evaluated by SYBR Green I fluorescence. The rat primers used for musclin wereforward, 5′-GGT GTC CTTG GAGA ATGATG-3′ and reverse, 5′-CGGTTTCTACCAATTCG ATC -3′, and those for β-actin were forward, 5′ – A TCT GGCACC ACA CCTTC-3′ and reverse, 5′-AG CCAG GTCCAGAC G CA-3′. All amplification reactions used the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA). After denaturation at 95 °C for 7 min, the solution was subjected to PCR for musclin at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s for 45 cycles.

Western blot analysis

Skeletal muscle tissues were homogenized in lysis buffer [0.1 mol/L NaCl, 0.01 mol/L Tris–HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L PMSF, 1%TritonX-100, 10 μg/ml pepstatin A and 500 KIU/mL aprotinin], and the homogenates were centrifuged at 3000 rpm for 15 min at 4 °C. Protein samples were separated using 10% SDS-PAGE and were then transferred to nitrocellulose for 3 h at 4 °C. The membranes were blocked with 5% nonfat, dried milk for 1 h at room temperature and were then incubated with the primary antibodies anti-β-actin, anti-GRP78, anti-GRP94, anti-musclin or anti-glucose transporter type-4 (GLUT-4) overnight at 4-8 °C. Then,the membranes were incubated for 1 h with the secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG). Protein expression was analyzed use NIH image analysis software (Bethesda, Maryland, USA) and normalized to β-actin expression. All experiments were repeated at least 3 times.

Statistical analysis

The data were analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA), and the results are expressed as the mean ± standard deviation. For continuous variables, comparisons between 2 groups were made using an unpaired Student t test, and those among more than 2 groups were made using one-way ANOVA, followed by Newman–Keuls multiple comparison test. Skewed data were analyzed using the Mann–Whitney U and Kruskal–Wallis H tests. Correlations between variables were determined using a simple linear regression analysis (Spearman's correlation). Unadjusted and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) predictive of subjects with overweight/obesity based on musclin level were generated using univariate and multivariate logistic regression analyses after controlling for other potential covariates. Values of $P < 0.05$ were considered significant.

Results

Subjects'characteristics

Demographic and laboratory characteristics stratified by BMI are presented in Table 1. Compared with the lean subjects, the individuals with overweight/obesity had higher diastolic blood pressure(DBP),TG,fasting serum insulin and HOMA-IR levels. There were no significant differences between the two groups in the other parameters, including age, fasting plasma glucose (FPG), TC, LDL-C and HDL-C.

Elevated plasma musclin levels in subjects with overweight/obesity

The musclin data were normally distributed. We found higher plasma musclin levels in subjects with overweight/obesity than in lean subjects (89.49 ± 19.00 ng/L vs 80.39 ± 16.35 ng/L, $P < 0.01$) (Fig. 1 and Table 1).

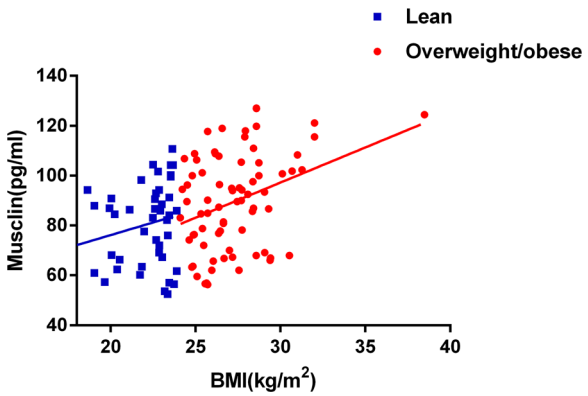

Fig. 1 Comparison of plasma musclin levels in lean controls and subjects with overweight/obesity

Relation between plasma musclin levels and overweight/obesity parameters

The Spearman correlation coefficients for associations between musclin concentrations and other parameters are summarized in Table 2. In all subjects, plasma musclin levels were significantly positively associated with BMI($r = 0.327$, $P < 0.01$) and TG($r = 0.191$, $P < 0.05$) but negatively associated with HDL($r = -0.282$, $P < 0.01$). In lean subjects, plasma musclin levels were not associated with BMI, DBP, SBP, or the other variables mentioned above. However, in subjects with overweight/obesity, plasma musclin levels were significantly positively associated with BMI ($r = 0.287$, $P < 0.05$) (Fig. 1), TG ($r = 0.237$, $P < 0.05$), FPG ($r = 0.314$, $P < 0.01$) and HOMA-IR ($r = 0.250$, $P < 0.05$) levels but negatively associated with HDL ($r = -0.318$, $P < 0.05$) (Fig. 2).

Correlation between overweight/obesity and the musclin level

The ORs of the musclin level being predictive of subjects with overweight/obesity were determined using univariate and multivariate logistic regression analyses; the results

Table 1 Subjects' characteristics

Variable	Lean ($n = 44$)	Overweight/obese ($n = 73$)	*P* value
Age (years)	51.75 ± 13.8	47.89 ± 9.8	0.08
Male/female	21(47.73%)/23	48(65.75%)/25	0.055
T2DM history	28(63.64%)	48(65.75%)	0.816
Current smoker (%)	9(20.45%)	26(35.62%)	0.083
Alcohol use (%)	14(31.82%)	31(42.47%)	0.252
Waist-to-hip ratio	0.9 ± 0.04	0.9 ± 0.05	0.491
Systolic blood pressure (mmHg)	127.36 ± 17.73	132.05 ± 16.73	0.154
Diastolic blood pressure (mmHg)	82.59 ± 9.43	87.68 ± 9.02	0.004**
Total cholesterol (mmol/L)	5.06 ± 1.01	5.36 ± 1.16	0.155
Triglycerides (mmol/L)	1.69 ± 1.41	2.55 ± 2.24	0.025*
High-density lipoprotein (mmol/L)	1.33 ± 0.29	1.26 ± 0.27	0.157
Low-density lipoprotein (mmol/L)	3.07 ± 0.84	3.2 ± 0.81	0.407
Fasting plasma glucose (mmol/L)	7.28 ± 3.29	8.18 ± 3.7	0.186
Fasting serum insulin (mmol/L)	8.46 ± 5.92	13.44 ± 8.70	0.001**
HOMA-IR (μIU x mol/L)	3.02 ± 3.94	5.42 ± 5.19	0.009**
Hemoglobin A_{1c} (%)	6.94 ± 2.32	7.54 ± 2.28	0.173
Musclin(ng/L)	80.39 ± 16.35	89.49 ± 19.00	0.009**

Data are presented as the mean ± standard deviation or as proportions (%)
*$P < 0.05$ vs. controls, **$P < 0.01$ vs. controls
T2DM type 2 diabetes mellitus, *HOMA-IR* homeostasis model assessment of insulin resistance

Table 2 Correlations of plasma musclin with anthropometric parameters

Variable	Total (n = 117)		Lean (n = 44)		Overweight/obese (n = 73)	
	r	P	r	p	r	p
Body mass index (kg/m^2)	0.327	0.001**	0.142	0.356	0.287	0.014*
Waist-to-hip ratio	−0.077	0.410	−0.050	0.746	−0.150	0.204
Systolic blood pressure (mmHg)	−0.097	0.299	−0.080	0.607	−0.167	0.157
Diastolic blood pressure (mmHg)	0.009	0.926	−0.006	0.967	−0.080	0.499
Total cholesterol (mmol/L)	−0.009	0.924	−0.202	0.188	0.044	0.709
Triglycerides (mmol/L)	0.191	0.039*	−0.019	0.903	0.237	0.044*
High-density lipoprotein (mmol/L)	−0.282	0.002**	−0.150	0.331	−0.318	0.006**
Low-density lipoprotein (mmol/L)	−0.120	0.197	−0.132	0.394	−0.156	0.189
Fasting plasma glucose (mmol/L)	−0.158	0.088	−0.174	0.258	0.314	0.007**
Fasting serum insulin (mmol/L)	0.089	0.342	−0.199	0.194	0.147	0.215
HOMA-IR (μIU x mol/L)	0.13	0.164	−0.292	0.054	0.25	0.033*
Hemoglobin A_{1c} (%)	0.028	0.766	−0.300	0.052	0.172	0.146

Correlations were determined using Spearman correlations
HOMA-IR homeostasis model assessment of insulin resistance. *P < 0.05, **P < 0.01

are shown in Table 3. In the univariate logistic regression analyses, the OR and 95% CI were 1.029(1.006-1.051) (P = 0.011). In model 1 of the multivariate logistic regression analyses, after adjusting for age and gender, the OR and 95% CI were 1.027(1.005-1.051) (P = 0.017). In model 2, after adjusting for age, gender, SBP, LDL-C, TC, insulin and HbA1c, the OR and 95% CI were 1.032(1.006-1.059) (P = 0.015). In model 3, after adjusting for age, gender, SBP, LDL-C, HDL-C, TC, TG, insulin, HbA1c and FPG, the OR and 95% CI were 1.033(1.005-1.061) (P = 0.019).

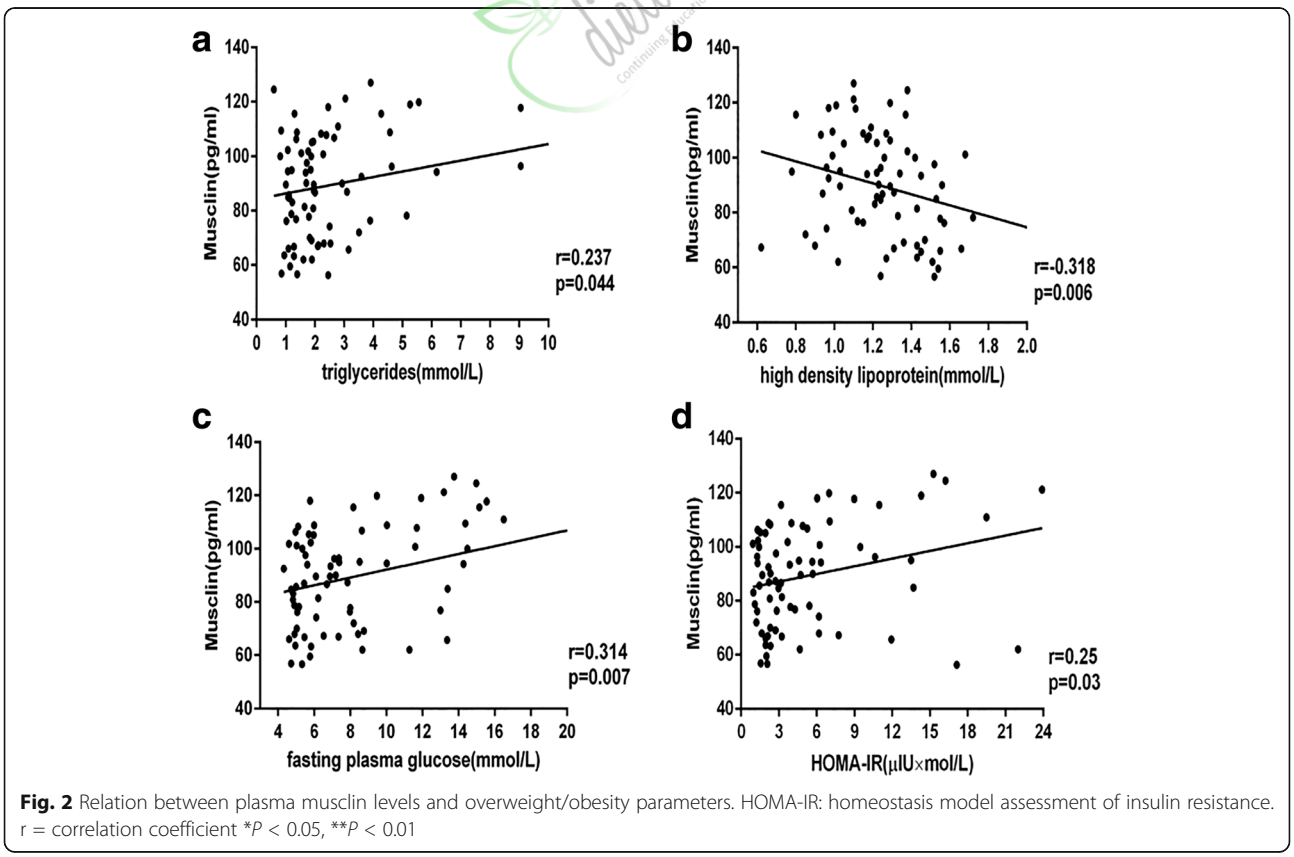

Fig. 2 Relation between plasma musclin levels and overweight/obesity parameters. HOMA-IR: homeostasis model assessment of insulin resistance. r = correlation coefficient *P < 0.05, **P < 0.01

Table 3 Univariate and multivariate logistic regression analyses of musclin predicting overweight/obese patients (n = 117)

Variable	*P*	OR	95% CI
Univariate	0.011	1.029	1.006-1.051
Multivariate			
Model 1[a]	0.017	1.027	1.005-1.051
Model 2[b]	0.015	1.032	1.006-1.059
Model3[c]	0.019	1.033	1.005-1.061
Model4[d]	0.031	1.031	1.003-1.060

Odds ratios(ORs) for overweight/obesity were calculated by logistic regression models. *CI* confidence interval
[a]Adjusted for age and gender
[b] Adjusted for age, gender, systolic blood pressure, low-density lipoprotein, total cholesterol, insulin and hemoglobin A_{1c}
[c]Adjusted for age, gender, systolic blood pressure, low-density lipoprotein, high-density lipoprotein,total cholesterol, triglycerides, insulin, hemoglobin A_{1c}, and fasting plasma glucose
[d] Adjusted for age, gender, systolic blood pressure, low-density lipoprotein, high-density lipoprotein,total cholesterol, triglycerides, insulin, hemoglobin A_{1c}, fasting plasma glucose, smoking and drinking history

In model 4, after adjusting for age, gender, SBP, LDL-C, HDL-C, TC, TG, insulin, HbA1c, FPG, smoking and drinking history, the OR and 95% CI were 1.031(1.003-1.060) (P = 0.031);

Insulin sensitivity was impaired in HFD rats

After 20 weeks, the body weight of the HFD rats increased by 13.49% (P < 0.01), the Lee index increased by 4.88% (P < 0.01) and the fat mass/body weight (%) increased by 89.32% (P < 0.01) (Table 4). The 20-weeks HFD also increased TC (by 24.79%), TG (by 27.78%) and LDL (by 49.60%) levels(all P < 0.05) but decreased the HDL level by 52.78% (P < 0.01) (Table 4). Insulin sensitivity was impaired in HFD rats, as shown by the HOMA-IR level,which increased by 113.45% (P < 0.01) (Additional file 1: Table S1). Compared with the control rats, the HFD increased serum insulin and FBG levels in rats by 76.68% (P < 0.01) and 37.6%(P < 0.01), respectively (Table 4). The glucose response during the OGTT in the HFD group was markedly increased by 50% (P < 0.01), 43% (P < 0.01), 34% (P < 0.01), 28% (P < 0.05) and 31% (P < 0.05) at the 0, 30, 60, 90, and 120 min time points, respectively, compared with those in the ND group (Fig. 3a). The total area under the glucose curve was also significantly increased by 36% (P < 0.05) in the HFD group (Fig. 3b). To accurately examine IR, we measured insulin-induced 2-DG uptake in the soleus muscles. In the HFD rats, 2-DG uptake (glucose transport activity) showed a 26.3% (P < 0.05) decrease compared with that in the ND rats (Fig. 3c).

Table 4 Comparison of plasma biochemical indicators in HFD and ND rats

Parameters	ND	HFD
Body weight (g)	546.30 ± 16.29	620.01 ± 13.31**
Lee index	301.27 ± 1.73	315.97 ± 2.26**
Fat mass/body weight (%)	4.12 ± 0.29	7.80 ± 0.72**
Triglyceride (mmol/L)	0.72 ± 0.04	0.92 ± 0.05*
Total cholesterol (mmol/L)	2.42 ± 0.10	3.02 ± 0.19*
High-density lipoprotein (mmol/L)	1.08 ± 0.10	0.51 ± 0.03**
Low-density lipoprotein (mmol/L)	1.25 ± 0.11	1.87 ± 0.22*
Fasting serum insulin(uIU/mL)	11.32 ± 0.46	20.0 ± 1.77**
Fasting blood glucose(mmol/L)	5.46 ± 0.09	7.51 ± 0.28**
HOMA-IR (μIU x mol/L)	2.75 ± 0.14	5.87 ± 0.67**

HFD high-fat diet, *HOMA-IRI* homeostasis model assessment of insulin resistance, *ND* normal diet. The data are shown as the mean ± standard error of the mean of each group; (n = 8); * P < 0.05 vs ND group. **P < 0.01 vs ND group

Musclin expression and its circulating levels increased in HFD rats

In the rat gastrocnemius muscles, the HFD increased musclin mRNA expression by 220% (P < 0.01) (Fig. 4a) and increased the musclin protein level by 52.8% (P < 0.05) and 67.70% (8.25 ± 0.45 ng/g vs 13.84 ± 2.16 ng/g,P < 0.05), as determined by Western blot and radioimmunoassay analyses, respectively (Fig. 4b,c). Compared with the control group, musclin immunoreactivity (musclin-ir) in plasma was increased in HFD rats (78.34 ± 7.52 ng/L vs 119.6 ± 6.71 ng/L,P < 0.01) (Fig. 4d).

Correlation between musclin content and an indicator of insulin resistance in HFD rats

The plasma musclin-ir concentrations were positively correlated with FBG (r = 0.79, p < 0.05), serum insulin (r = 0.81, p < 0.05) and glucose uptake (r = 0.875, p < 0.05) of skeletal muscle in HFD rats (Fig. 5 and Additional file 1: Table S1). There was no significant correlation between plasma musclin content and obese indicators including weight, Lee index and fat mass/body weight. The skeletal muscle musclin-ir displayed a positive correlation with FBG (r = 0.901, p < 0.01), serum insulin (r = 0.879, p < 0.01) and glucose uptake (r = 0.777, p < 0.05) of skeletal muscle in HFD rats (Fig. 6 and Additional file 2: Table S2). There was no significant correlation between skeletal muscle musclin content and obese indicators including weight, Lee index and fat mass/body weight.

Musclin induced IR and inhibited the protein expression of GLUT-4 in rat skeletal muscle

To determine whether musclin impaired the glucose uptake ability of skeletal muscle, we tested the effect of musclin on 2-DG uptake in the soleus muscles. We observed a 48.3% (P < 0.01) decrease in 2-DG uptake in the ND group after incubation with musclin (1.5×10^{-7} mol/L) compared with the control, while there was no significant decreasein 2-DG uptake in the HFD group after incubation with musclin (Fig. 7a). Compared with

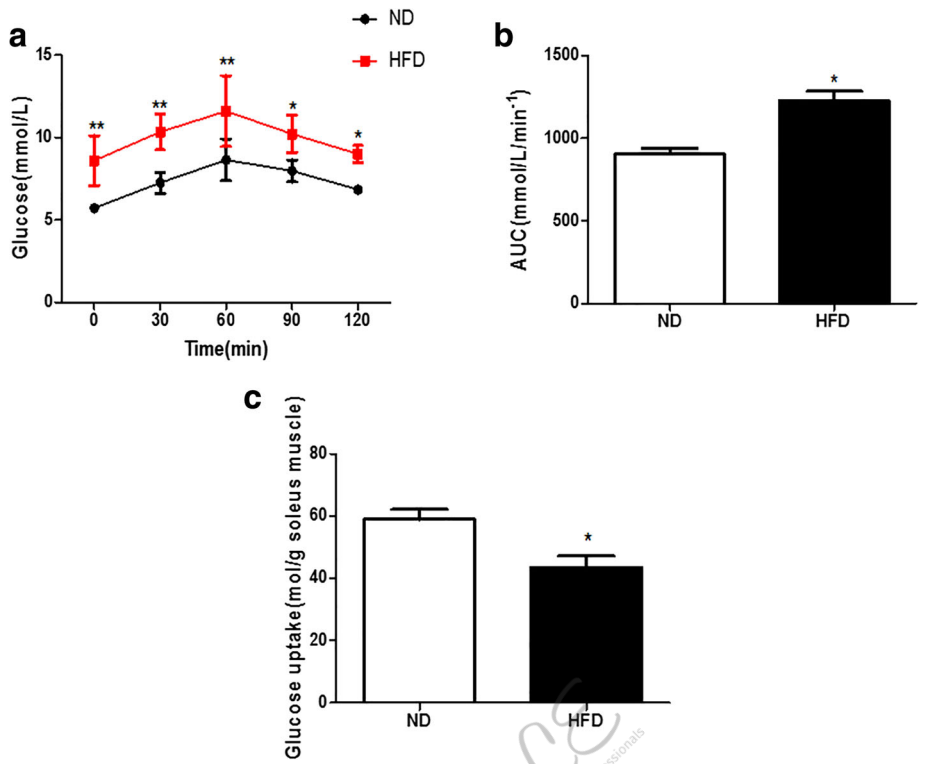

Fig. 3 Twenty-week high-fat diet induced insulin resistance. **a**: Mean blood glucose levels during the oral glucose tolerance test; **b**: Total area under the glucose curve. **c**: Effect of high-fat diet on glucose uptake in soleus muscles. ND: normal diet; HFD: high-fat diet; AUC: area under the curve. Values are the mean ± standard error of the mean; (n = 8); *P < 0.05 vsND,**P < 0.01 vs ND

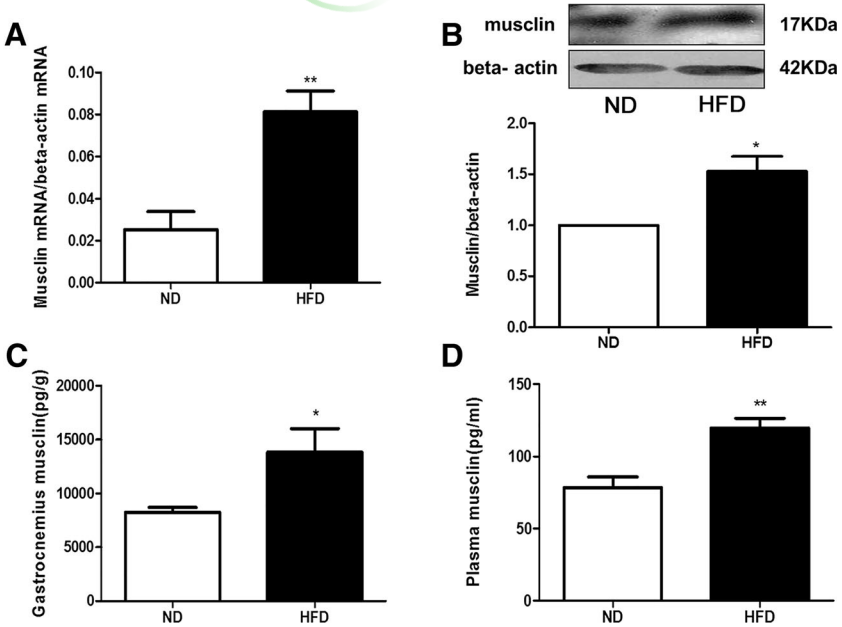

Fig. 4 Musclin expression and its circulating musclin levels increased in HFD rats. **a**: Real-time PCR analysis of musclin expression in gastrocnemius muscles. **b**: Western blot analysis of musclin protein expression in gastrocnemius muscles and ratio of musclin to β-actin determined by quantitative analysis. **c**: Radioimmunoassay analysis of musclin content in gastrocnemius muscles. **d**: Radioimmunoassay analysis of plasma musclin levels. ND: normal diet; HFD: high-fat diet. Values are the mean ± standard error of the mean; (n = 8); *P < 0.05 vs ND

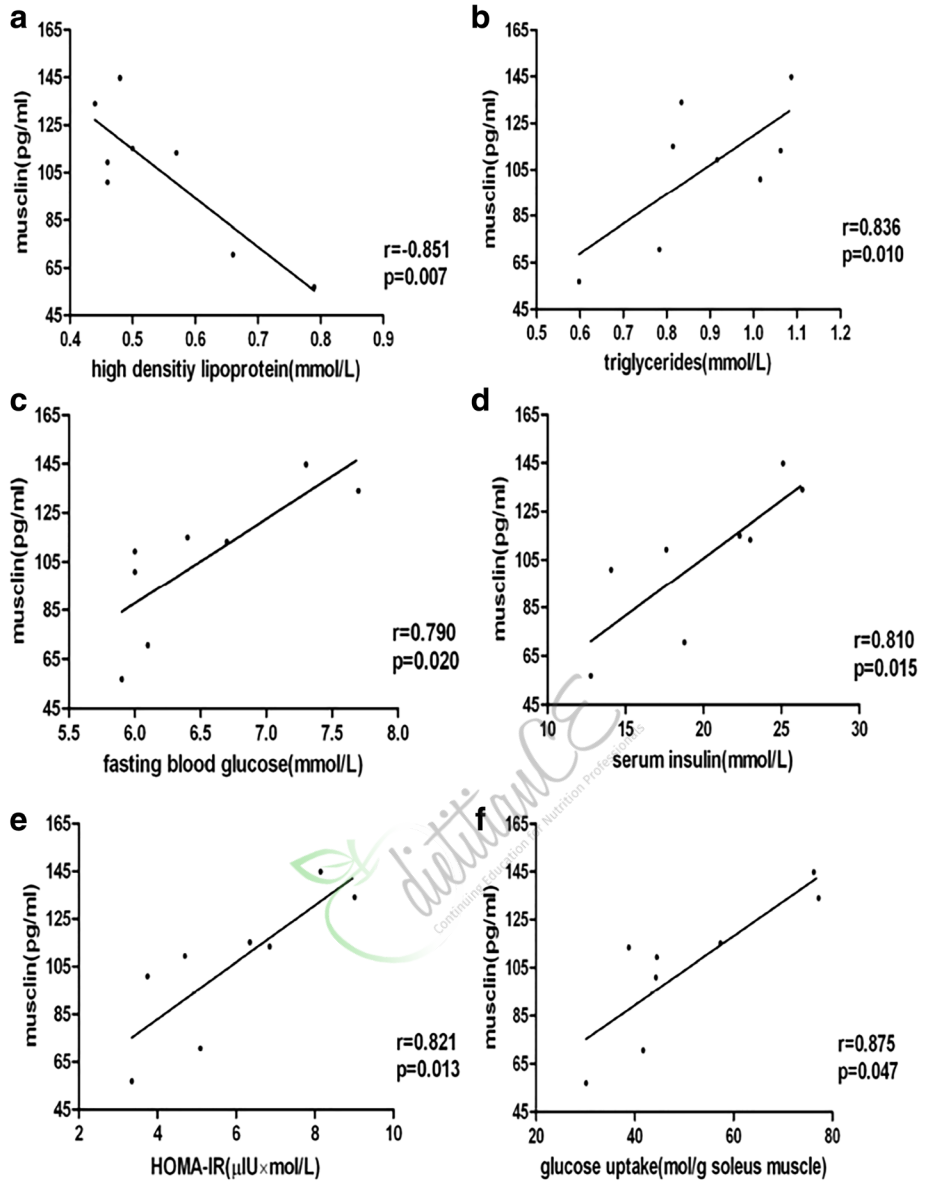

Fig. 5 Correlation between plasma immunoreactive musclin concentrations and plasma biochemical indicator in HFD rats. HOMA-IR: homeostasis model assessment of insulin resistance. r = correlation coefficient *$P < 0.05$, **$P < 0.01$

the ND rats, the HFD rats exhibited a marked decrease in the GLUT-4 protein level ($P < 0.05$). The protein expression of GLUT-4 in the soleus muscle was significantly downregulated by 30% ($P < 0.05$) in the ND group after incubation with musclin compared with the control (Fig. 7b,c).

Musclin increased skeletal muscle endoplasmic reticulum stress (ERS) marker levels

After 20 weeks, the HFD rats showed GRP78 and GRP94 protein levels in skeletal muscle that were significantly increased by 136.1 and 48.6% (both $P < 0.05$), respectively, compared with the ND rats. The Western blot analysis showed thatinthe ND group, muscle incubation with musclin increased the protein levels of GRP78 and GRP94 by 146.8 and 54% (both $P < 0.05$), respectively. However, in the HFD group, there were no differences in GRP78 and GRP94 protein expression after the musclin incubation (Fig. 8).

Discussion

The present study demonstrated for the first time that plasma musclin levels were significantly higher in subjects with obesity than in lean controls. In addition, the plasma musclin concentration was positively correlated with IR-related laboratory parameters as well as fasting

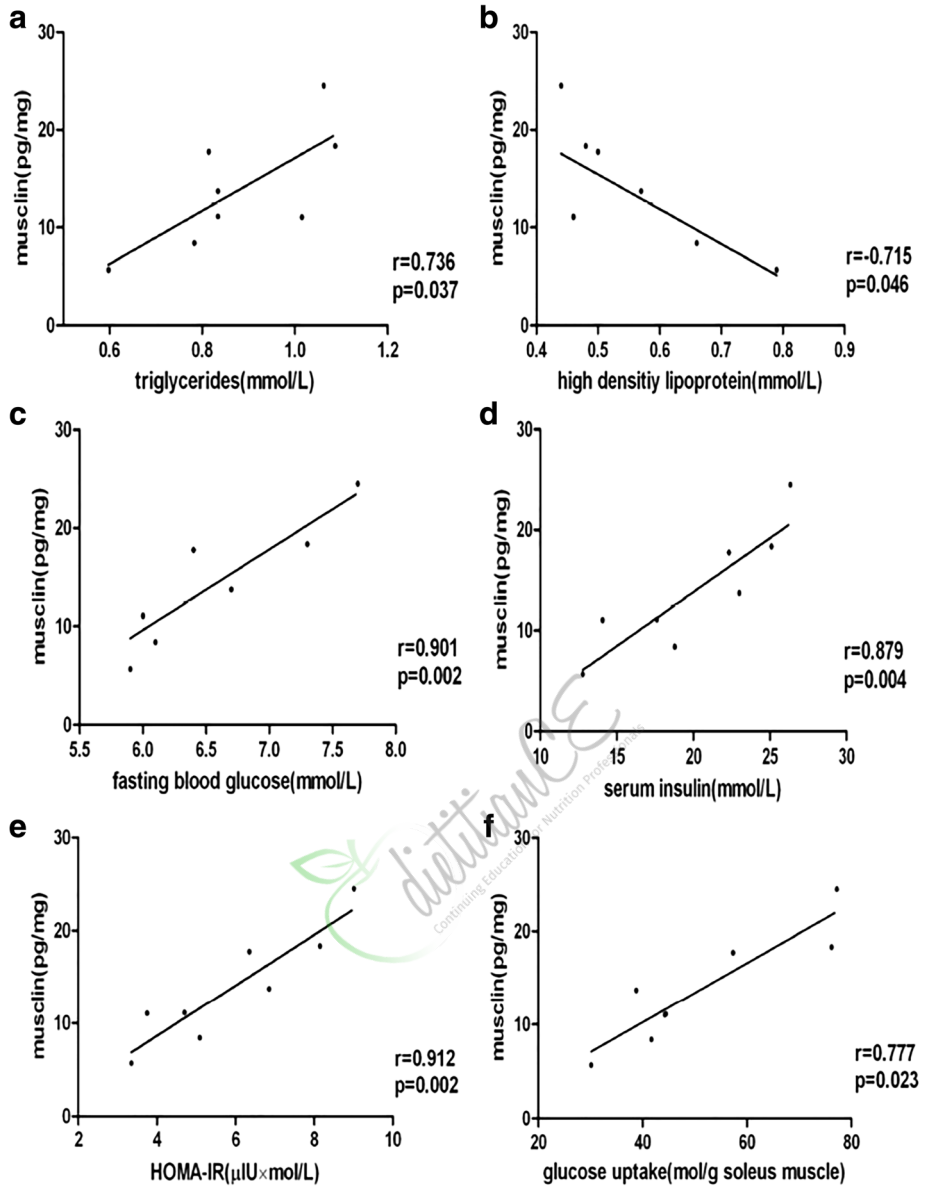

Fig. 6 Correlation between skeletal muscle immunoreactive musclin concentrations and plasma biochemical indicator in HFD rats. HOMA-IR: homeostasis model assessment of insulin resistance. r = correlation coefficient *$P < 0.05$, **$P < 0.01$

glucose and HOMA-IR levels. Furthermore, the human study results were supported by the findings of the animal experiments, which showed that rats with obesity-associated IR had markedly increased plasma and skeletal muscle musclin expression. Using ex-vivo assays, we also found that musclin impaired insulin-induced glucose uptake and inhibited the protein expression of GLUT-4 related to the activation of ERS. This alteration of endogenous musclin expression in skeletal muscle and plasma in humans and rats with obesity-associated IR has never been previously reported.

Obesity-induced IR is a key pathophysiological feature of T2DM; however, the specific mechanism linking IR and obesity has not been established before. In our study, plasma musclin levels were investigated in subjects with overweight/obesity (BMI > 24 kg/m^2), and we demonstrated for the first time that musclin plasma concentrations were significantly increased in the subjects with overweight/obesity compared with the lean subjects. Our data showed that the subjects with overweight/obesity had higher insulin and HOMA-IR levels, suggesting that the subjects with overweight/obesity had obtained IR. In addition, we found that the musclin levels in the subjects with overweight/obesity were significantly correlated with markers of obesity and IR, such as BMI, HOMA-IR and FBG levels. Hence, we

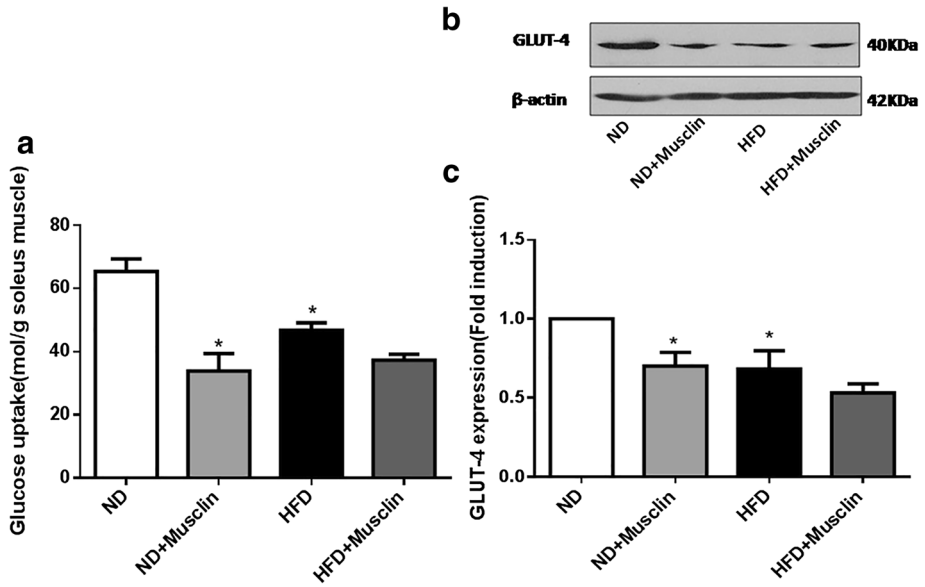

Fig. 7 Incubating soleus muscles with musclin induced IR and inhibited the protein expression of GLUT-4. **a**: Glucose transport activity (2-DG) in soleus muscles. **b**: Western blot analysis of the protein level of 40-kDa GLUT-4 in soleus muscles. **c**: Quantitative analysis of GLUT-4 protein expression. GLUT-4:glucose transporter type 4. Values are the mean ± standard error of the mean; (n = 3); *P < 0.05 vsND,**P < 0.01 vs ND

speculate that musclin may play an important role in obesity-associated IR.

We then performed a further study in rats with obesity-associated IR induced by a HFD. After 20 weeks, the HFD rats exhibited dyslipidemia, as indicated by higher serum TC and TG levels, similar to what is observed in human obesity. IR leads to hyperinsulinemia and a decreased glucose metabolism [15]. Our results also show that the obese rats exhibited characteristics of IR, such as elevated blood glucose levels, hyperinsulinemia and impaired

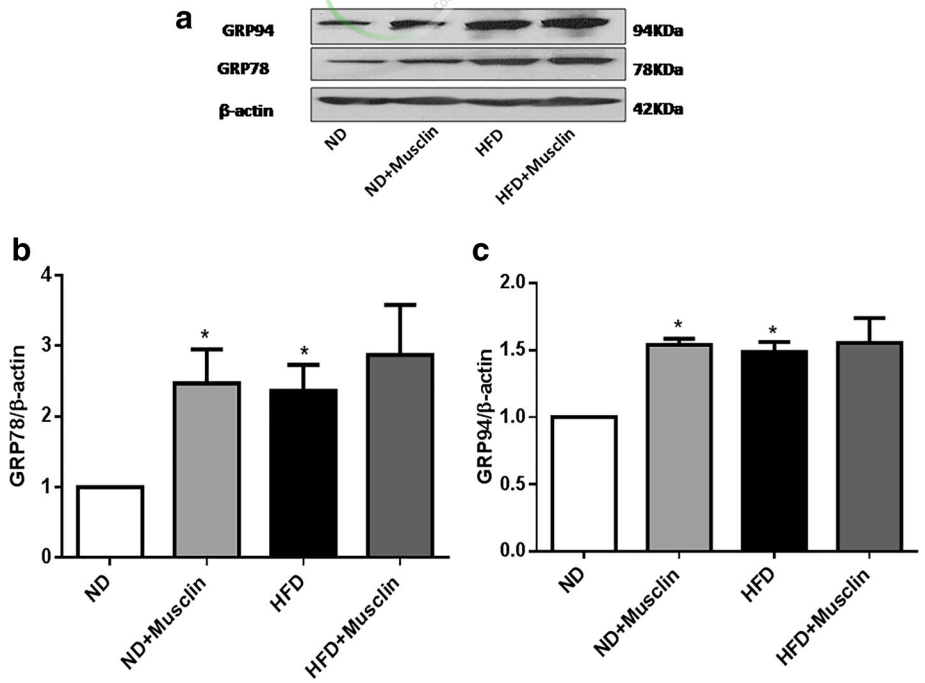

Fig. 8 Effect of musclin on ERS markers in rat soleus muscles. **a**: Western blot analysis of the protein levels of the 78-kDa glucose-regulated protein (GRP78) and the 94-kDa GRP94 in soleus muscles. **b**: Quantitative analysis of GRP78 protein expression. **c**: Quantitative analysis of GRP94 protein expression. Values are the mean ± standard error of the mean; (n = 3); *P < 0.05 vsND,**P < 0.01 vs ND

glucose tolerance. Additionally, the glucose uptake by skeletal muscle with IR was significantly decreased, which is in agreement with the findings of previous reports [16]. Recent research has shown that myofibers produce and release musclin in a fiber-type-specific manner, wherein higher levels of musclin are found in the fast-twitch plantaris and white gastrocnemius muscles, especially in the type IIb fibers [17]. In the present study, we used white gastrocnemius rat musclesto to investigate the expression of musclin. We found that the gene expression of musclin was clearly upregulated in the HFD rat skeletal muscles, and the musclin protein levels were also significantly increased, as determined by Western blot and radioimmunoassay analyses. Several studies have demonstrated increased musclin levels is associated with metabolic disorder. Nishizawa et al. reported that musclin mRNA expression was augmented by approximately 4-fold in the gastrocnemius muscles of obese KKAy mice and db/db mice [9]. Yu et al. found musclin expression was significantly elevated in the skeletal muscle of HFD rats [18]. Chen et al. reported increased circulating levels of musclin in newly diagnosed T2DM patients [19]. In our study, we demonstrated for the first time that the plasma musclin-ir was up regulated in rats with obesity-associated IR. We then determined the causes of the higher musclin plasma levels in HFD rats. Compared with the ND rats, the HFD rats showed skeletal muscle musclin mRNA expression that was increased by 2-fold. In addition, in the HFD group, the musclin expression in the skeletal muscles was approximately 100-fold higher than that in the plasma. Based on a previous report demonstrating that musclin is exclusively expressed in skeletal muscles [9], together with the significantly higher musclin expression in skeletal muscles, the findings of the current study suggest that the increased plasma musclin in rats with IR might be secreted predominantly by skeletal muscles.

A few investigations have addressed the mechanism and regulation of musclin expression in the state of IR. Our results suggested that incubating the skeletal muscles of ND rats with musclin could induce IR, leading to decreased 2-DG uptake. A recent study has shown that the preincubation of skeletal muscles with musclin caused decreased insulin-stimulated 2-DG uptake and decreased Akt/PKB activation in the insulin-signaling cascade [10]. In our study, we also found that musclin suppresses the expression of GLUT-4 protein in ND rats. These results suggest that musclin could exert effects on glucose homeostasis that may be mediated via changes in the insulin sensitivity of skeletal muscle.

Notably, our study demonstrated that musclin caused ERS in skeletal muscle. In recent years, ERS has been implicated in the development of peripheral IR, obesity and T2DM [20]. Deldicque et al. showed that a 20-weeks HFD increased the protein and mRNA levels of factors involved in the unfolded protein response, such as binding protein/GRP78, p-protein kinase R-like ER protein kinase, CHOP and inositol-requiring enzyme 1α in skeletal muscle [21]. Gu et al. found that palmitate can induce a high expression of musclin in C2C12 myotubes, and that the PERK signaling pathway is potentially involved in this process [22]. In our study, we also found that ERS markers, such as GRP78 and GRP94,were markedly increased in the skeletal muscles of the HFD rats, which was consistent with the findings of previous studies. Furthermore, we also found that musclin incubation increased the protein levels of GRP78 and GRP94.Therefore, these results demonstrated that musclin could induce IR, at least in part, through causing ERS in skeletal muscles.

Conclusion

In summary, we showed that HFD upregulated the expression of endogenous musclin in skeletal muscles and plasma in obesity-associated IR. Treating skeletal muscle with musclin induced IR and impaired the glucose metabolism, at least in part, through causing ERS. Musclin is an important myokine that participates in the development of skeletal muscle IR and the regulation of peripheral glucose homeostasis in subjects with obesity and HFD rats. Further research is warranted to investigate the functional role of musclin in the development of obesity-associated IR and its mechanism of regulation.

Effects of resveratrol on glucose control and insulin sensitivity in subjects with type 2 diabetes: systematic review and meta-analysis

Abstract: Although the regular consumption of resveratrol has been known to improve glucose homeostasis and reverse insulin resistance in type 2 diabetes mellitus (T2DM), the reported results are inconsistent. Thus, we aimed to assess the effects of resveratrol on glycemic control and insulin sensitivity among patients with T2DM. We searched for relevant articles published until June 2017 on PubMed-Medline, Embase, Cochrane Library, and Web of Science. Randomized controlled trials in T2DM patients administered with resveratrol as intervention were included. After study selection, quality assessment and data extraction were performed independently by two authors, and STATA and RevMan software were used for statistical analysis. Nine randomized controlled trials involving 283 participants were included. Meta-analysis showed that resveratrol significantly improved the fasting plasma glucose (−0.29 mmol/l, 95% CI: −0.51, −0.06, $p < 0.01$) and insulin levels (−0.64 U/mL, 95% CI: −0.95, −0.32, $p < 0.0001$). The drug also reduced homeostasis model assessment of insulin resistance (HOMA-IR) index, systolic blood pressure, and diastolic blood pressure among participants with T2DM. The changes in hemoglobin A1c (HbA1c), low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were negligible. Subgroup analysis comparing the resveratrol supplementation doses of < 100 mg/d versus ≥ 100 mg/d revealed a significant difference in fasting plasma glucose. In particular, the latter dose presented more favorable results. This meta-analysis provides evidence that supplementation of resveratrol may benefit management of T2DM.

Keywords: Resveratrol, Type 2 diabetes, Meta-analysis

Background

Type 2 diabetes mellitus (T2DM) is a long-term, multifactorial, metabolic disease with severe complications. Projections indicate that approximately 600 million people will suffer from the disease by 2030 [1, 2]. Increasing morbidity and mortality rates among patients with T2DM are mainly attributed to the high incidence and severity of diabetic complications. These complications pose a major threat to general public health worldwide and lead to high economic costs [3, 4]. Hence, identifying an optimal therapy for T2DM is crucial. Although chemical agents for glycemic control have been adopted in T2DM therapy, these substances are limited by their contraindications and side effects, especially hypoglycemia and weight gain [5], which require an effective treatment method for T2DM.

As a potent antioxidant, resveratrol is a plant-derived polyphenolic compound that possesses anti-inflammatory, antiplatelet aggregation, anti-carcinogenic, cartilage-protective, and anti-aging properties. This compound also improves the endothelial function [6]. Some studies have revealed that resveratrol administration improves insulin sensitivity in diabetic rats and patients with T2DM [7–9]. In vitro and in vivo studies have described resveratrol as a potent activator of histone deacetylase Sirtuin1 (Sirt1) [10, 11]. Sirt1 activation can increase insulin sensitivity and protect against metabolic damage resulting from a high-fat diet. AMP-activated protein kinase (AMPK) activation has been used to mediate some effects of resveratrol in regulating insulin sensitivity and insulin secretion in

pancreatic β-cells and increasing glucose uptake [12, 13]. These results indicate that resveratrol is an inexpensive dietary supplement that could benefit T2DM treatment.

Resveratrol was added to the usual therapy in trials, which revealed improvements in glycemic control, insulin sensitivity, and other metabolic parameters of patients with T2DM [14, 15]. However, inconsistent results were on the therapeutic efficacy of resveratrol treatment from several human clinical trials [16, 17]. According to a recent meta-analysis, resveratrol supplementation is more effective than placebo in terms of hemoglobin A1c (HbA1c) and creatinine levels, but this finding is not true for fasting plasma glucose and insulin resistance in patients with T2DM [18]. Furthermore, the evidence supporting the beneficial effects of resveratrol in T2DM treatment is contradicting. In the present work, we therefore performed a meta-analysis of randomized controlled trials (RCTs) to determine whether or not consuming resveratrol could modulate blood glucose homeostasis and improve insulin sensitivity as compared with placebo/control in patients with T2DM.

Methods

This study follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [19]. The PRISMA statement is designed to improve the quality of meta-analyses.

Literature search

The databases of PubMed-Medline, Embase, Cochrane Library, and Web of Science were searched for RCTs that were published until June 2017 and evaluated the effects of resveratrol treatment versus placebo/control on T2DM. The following search strategy combined free keywords with MeSh terms: [resveratrol or polygonum or polyphenolic compound or red wine or red grapes or knotweed or SRT50] AND [diabetes or diabetic]. Only English language articles were included. A historical search was also performed using the reference lists of relevant articles. The detailed search strategy is presented in Additional file 1: Table S1.

Study selection

Eligible studies were determined by two reviewers (XYZ, CHW), and disagreement was resolved by discussion and consultation with a third reviewer (SHQ). Inclusion criteria were as follows: (1) published research articles with completed RCTs reported by original articles, (2) participants suffering from T2DM, (3) studies comparing the effects of resveratrol at any dosage with those of placebo/control, and (4) articles on fasting plasma glucose or HbA1c or homeostasis model assessment of insulin resistance (HOMA-IR). Exclusion criteria were as follows: (1) duplicated publications, and only the first publication reporting related outcomes was included; (2) trials involving animals or healthy human subjects; (3) non-randomized trials; (4) case reports or series studies; and (5) articles that provided inadequate information of interest or primary data.

Data abstraction and quality assessment

The following details of each included trial were extracted to identify the effects of resveratrol on glycemic control: first author's name, publication year, study design, patient quantity, resveratrol dose, study duration, and outcome measures. The baseline and end point information on glucose parameters, including fasting plasma glucose, fasting insulin, HbA1c, and HOMA-IR, were also extracted. We recorded other indicators (e.g., baseline and end point and changes in systolic blood pressure, diastolic blood pressure, low-density lipoprotein cholesterol [LDL-c], and high-density lipoprotein cholesterol [HDL-c] levels) to thoroughly understand the relationship between cardiometabolic risk indicator and glycemic control. All values were changed to mmol/L for glucose and to pmol/L for insulin by using the conversion factors 1 mmol/L = 18 mg/dL and 1 pmol/L = 6.965 mIU/L, respectively.

We evaluated the bias risk for each study by using the Cochrane tool (Higgins & Green, 2011), which includes random sequence generation, allocation concealment, blinding (participant, personnel, and outcome assessment), incomplete outcome data, selective outcome reporting, and other biases. The judgment of authors is categorized as "Low risk", "High risk" or "Unclear risk" of bias.

Publication bias

According to Egger and colleagues, publication bias assessment is not reliable for less than 10 pooled studies [20]. Therefore, in the present study, we could not assess the existence of publication bias by Egger's test for funnel plot asymmetry.

Statistical analysis

The primary outcomes were fasting plasma glucose, HbA1c, and HOMA-IR. The secondary outcomes included insulin concentration, systolic blood pressure, diastolic blood pressure, LDL-c, and HDL-c. I^2 statistic and Cochrane's Q were used to identify heterogeneity among the studies [21]. In case of heterogeneity (Cochrane's Q $p < 0.10$ or $I^2 > 50\%$), the data were pooled using a random-effect model. Otherwise, the fixed-effect model was used. We calculated the mean differences between resveratrol and placebo/control groups by using the standardized mean difference (SMD) and 95% confidence interval (CI). We extracted the means and standard deviations (SDs) for the baseline and post-

treatment for both groups when available. When the means and SDs were unavailable, we extracted the change scores. Subgroup analysis was conducted for fasting plasma glucose in accordance with the following criteria: resveratrol dose at < 100 versus ≥ 100 mg/d; and treatment duration at < 12 weeks versus ≥ 12 weeks. In case of heterogeneity, we performed sensitivity analyses to test the robustness of the pooled estimates, by using the leave-one-out approach (i.e., removing one study each time and repeating the analysis). All the preceding analyses were performed using Stata 12.0 (Stata Corp, Texas, USA) and RevMan v5.2 software.

Results

Search results and study characteristics

The initial search yielded 973 potentially relevant articles. Titles and abstracts were screened, and 16 articles were retrieved [7, 14–18, 22–31]. Seven studies were further excluded because (1) two studies were not randomized controlled trials [18, 22], (2) two studies did not provide available outcome data [16, 23], and (3) three studies included subjects with impaired insulin sensitivity but without T2DM [7, 24, 25]. Nine trials involving 283 participants were included in the meta-analysis (Fig. 1) [14, 15, 17, 26–31]. Among these trials, six reported the mean and SD values of HbA1c, five reported the mean and SD values of insulin and HOMA-IR, and all nine reported the mean and SD values of fasting plasma glucose. The sample size range is 10–64 participants. Resveratrol dose ranged from 8 mg/d to 3000 mg/d, and the duration of intervention varied from 4 weeks to 12 months. The detailed characteristics of the studies are presented in Tables 1 and 2.

Meta-analysis and subgroup analyses of the effects on primary outcomes

Fasting plasma glucose

Nine clinical studies involving 283 participants were included in the analysis to investigate the effects of resveratrol on fasting plasma glucose. A fixed-effect model analysis (I^2 = 0.0%, p = 0.44) was performed to pool the data. The overall results of the meta-analysis showed that resveratrol significantly reduced fasting plasma glucose as compared with placebo/control in patients with T2DM (−0.29 mmol/l, 95% CI: −0.51, −0.06, p < 0.01) (Fig. 2).

The subgroup analyses showed that fasting plasma glucose was not improve by the low-dose resveratrol (−0.01 mmol/l; 95% CI: −0.39, 0.37; p = 0.96), whereas its level significantly declined among the subgroup who received a high dose of resveratrol (−0.44 mmol/l; 95% CI: −0.72, −0.16, p < 0.002). The pooled effects of resveratrol on the fasting glucose of the participants were not influenced by the study duration.

HbA1c

Six studies involving 228 patients reported HbA1c levels before and after intervention. The random-effects model was used because significant heterogeneity was detected (I^2 = 94.7%, p = 0.001). The pooled estimates of mean difference suggested no significant difference in the HbA1c level between the resveratrol and control groups (−1.10; 95% CI: −2.46, 0.26; p = 0.11) (Fig. 3). When the study by Bhatt et al. [14] was removed, the heterogeneity of study results on HbA1c became insignificant (I^2 = 44.6%, p = 0.13). The effect of resveratrol supplementation on HbA1c remained unchanged, thereby suggesting that the study quality does not affect this outcome. The changes in HbA1c level were insignificantly different between the two groups (−0.04; 95% CI: −0.48, 0.39; p = 0.13).

HOMA-IR

Data from five trials involving 153 patients reported the effect of resveratrol therapy on HOMA-IR. Random-effect model analysis (I^2 = 52.7%, p = 0.08) was performed to pool the data on HOMA-IR. Meta-analysis of these studies revealed that resveratrol significantly reduced HOMA-IR (−0.52; 95% CI: −1.00, −0.04; p < 0.0001) (Fig. 4).

Meta-analysis of the effects on secondary outcomes

Compared with those in the placebo/control groups, the patients with T2DM who received resveratrol supplementation achieved low insulin levels (−0.64 pmol/L; 95% CI: −0.95, −0.32; p < 0.0001), systolic blood pressure (−0.58 mmHg; 95% CI: −0.86, −0.30; p < 0.0001), and diastolic blood pressure (−0.43 mmHg; 95% CI: −0.70, −0.15; p < 0.003) after treatment. No significantly different effects were observed for LDL-c (−0.57 mmol/l, 95% CI: −2.10, 0.96, p = 0.46) and HDL-c (0.30 mmol/l; 95% CI: −0.45, 1.04; p = 0.43). An additional movie file shows this finding in detail (Additional file 2: Figure S1).

Risk of bias assessment

The overall risk of bias in the included trials was moderate. The reporting of allocation concealment was unclear in the majority of trials. Some studies did not provide sufficient information to determine the blinding of the participants, personnel, and outcome assessments. Several studies showed a low bias risk toward selective reporting, and two studies even exhibited a high bias risk in this regard. Three studies exhibited a high bias risk in incomplete outcome data. The quality of bias assessment for the included studies is described in Additional file 3: Figure S2.

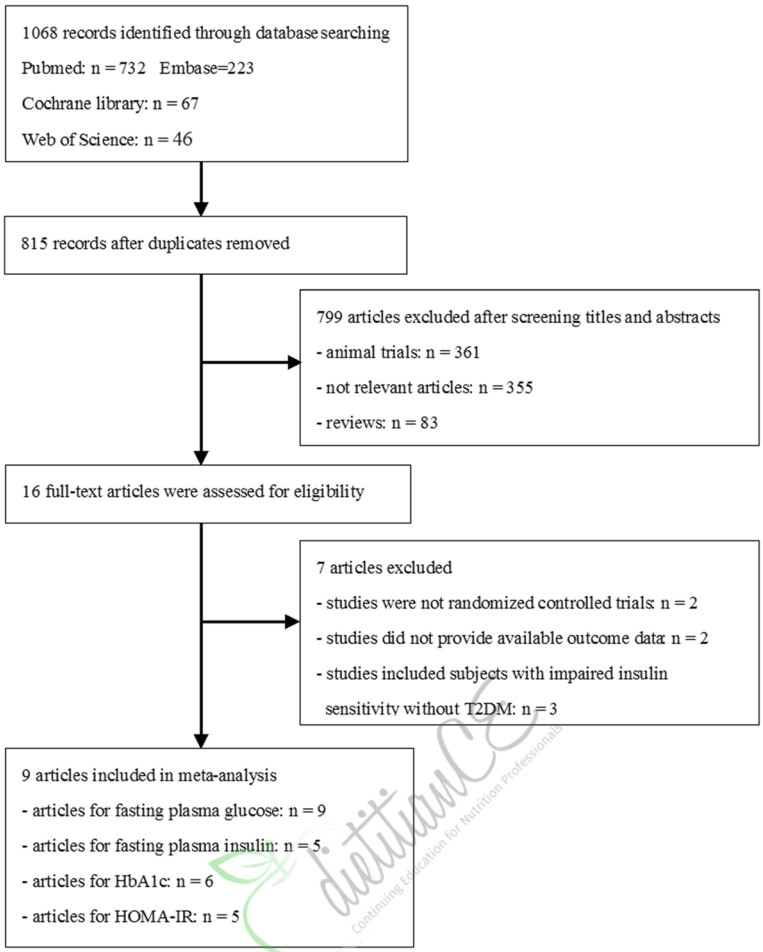

Fig. 1 Flow diagram of the study selection procedure showing the number of eligible articles included in the meta-analysis

Adverse events

Most studies did not mention adverse reactions during therapy. A major concern in using high dose of resveratrol is related to its toxic effects on the major organs in the body. However, Movahed et al. [28] and Thazhath et al. [17] reported that a maximum dose of 1 g/day of resveratrol is well tolerated and shows no toxic effects in patients with diabetes.

Discussion

As revealed in this review, resveratrol supplementation for T2DM treatment resulted in significant and clinically important changes in the levels of fasting plasma glucose and insulin, HOMA-IR index, systolic blood pressure, and diastolic blood pressure. However, we failed to show the positive effects on HbA1c, LDL-c, and HDL-c. Subgroup analyses showed a significant effect of high-dose resveratrol supplementation (≥ 100 mg/d) on reducing the fasting plasma glucose levels. By contrast, low-dose resveratrol supplementation (< 100 mg/d) showed no significant effects on fasting plasma glucose levels.

Meta-analysis results are consistent with those of a previous meta-analysis, which reported that resveratrol consumption significantly reduced the fasting glucose and insulin levels, and reversed insulin resistance among participants with diabetes [32]. However, only 3 of the 11 studies included in their review investigated the patients with T2DM. Hausenblas et al. [18] found six eligible studies, which were also included in the present review. In contrast to the findings of the present review, those of the previous review revealed that resveratrol consumption showed that resveratrol has insignificant effects on the fasting glucose and insulin levels but positive effects on HbA1c. However, such study included six trials with <200 participants, which possibly resulted in selection bias. The current systematic review provided additional data that were used to examine the effects of resveratrol treatment on clinically relevant metabolic parameters in patients with T2DM.

Reducing blood glucose levels is a highly important criterion for managing diabetes. Several clinical studies were performed to examine the effectiveness of resveratrol

Table 1 Characteristics of the 9 randomized controlled trials included in the analysis

Study	Study design	Population	Duration	Resveratrol group	Control group	Outcomes
Brasnyo et al.	Randomized placebo-controlled double-blinded parallel clinical trial	*N* = 19 patients with T2DM	4 weeks	*N* = 10;10 mg/d	*N* = 9; Placebo	Fasting plasma glucose, fasting insulin, HbA1c, HOMA-IR
Bhatt et al.	Open-label, randomized, controlled trial	*N* = 57 patients with T2DM	3 months	*N* = 28;250 mg/d	*N* = 29; Empty-control	Fasting plasma glucose, HbA1c
Movahed et al.	Randomized placebo-controlled double-blinded parallel clinical trial	*N* = 64 patients with T2DM	45 days	*N* = 33;1 g/d	*N* = 31; Placebo	Fasting plasma glucose, fasting insulin, HbA1c, HOMA-IR
Goh et al.	Randomized double-blind	*N* = 10 patients with T2DM	12 weeks	*N* = 5;3 g/d	*N* = 5; Placebo	Fasting plasma glucose, fasting insulin, HbA1c, HOMA-IR
Tome-Carneiro et al.	Randomized placebo-controlled triple -blinded parallel clinical trial	*N* = 35 patients with T2DM	12 months	*N* = 13; RSV-enriched grape extract (8 mg/d)	*N* = 22; Placebo	Fasting plasma glucose, HbA1c
Bashmakov et al.	Randomized placebo-controlled examiner -blinded parallel clinical trial	*N* = 24 patients with diabetic foot syndrome	60 days	*N* = 14; 50 mg/day	*N* = 10; Placebo	Fasting plasma glucose, fasting insulin
Thazhath et al.	Randomized, double-blind, crossover design	*N* = 14 patients with T2DM	5 weeks	*N* = 14;1 g/d	*N* = 14; Placebo	Fasting plasma glucose, HbA1c
Timmers et al.	Randomized double-blind crossover study	*N* = 17 patients with T2DM	30 days	*N* = 17;150 mg/d	*N* = 17; Placebo	Fasting plasma glucose, fasting insulin, HbA1c, HOMA-IR
Javid et al.	Randomized placebo-controlled double-blinded parallel clinical trial	*N* = 43 patients with T2DM	4 weeks	*N* = 21;480 mg/d	*N* = 22; Placebo	Fasting plasma glucose, fasting insulin, HOMA-IR

T2DM type 2 diabetes mellitus, *HOMA-IR* homeostasis model assessment of insulin resistance, *HbA1C* glycated hemoglobin A

Table 2 Baseline characteristics of the included studies

Study	Group	Age Mean (SD)	Sex No. of Female/Male	Weight Mean (SD)	BMI Mean (SD)	SBP Mean (SD)	FBG Mean (SD)	Duration– yr. Mean (SD)	Smoker – no. (%)
Brasnyo et al.	Resveratrol	57·9(7·9)	NA	90.1(16.3)	NA	140(12)	7.9(2.21)	NA	0(10)
	Placebo	52.5(11.1)		105.3(16.7)		140(17)	8.8(3.2)		0(9)
Bhatt et al.	Resveratrol	56.67(8.91)	16/12	64.78(9.25)	24.66(3.57)	139.71(16.10)	11.82(3.58)	7.57(4.56)	6/28
	Placebo	57.75(8.71)	20/9	63.1(9.02)	24.92(3.05)	134.51(14.61)	10.11(2.56)	6.68(4.7)	6/29
Movahed et al.	Resveratrol	52.45(6.18)	17/16	74.26(11.39)	27.05(3.13)	129.03(14.91)	9.76(2.76)	5.81(1.53)	7(21.9)
	Placebo	51.81(6.99)	16/17	76.60(14.27)	27.83(4.12)	129.31(15.16)	8.40(2.86)	5.39(1.36)	4.(12.9)
Goh et al.	Resveratrol	55.8(7.3)	NA	87.0(26.6)	29.4(6.8)	NA	11.8(2.9)	9.4(5.3)	1(10)
	Placebo	56.3(6.0)		68.3(13.7)	24.4(3.6)		9.5(1.0)	9.6(6.3)	2(20)
Tome-Carneiro et al.	Resveratrol	63(12)	NA	84(11)	31(5.1)	130(16)	8.39(3)	NA	2(22)
	Placebo	58(10)		85(16)	32(4.5)	129(21)	8.22(2)		8(36)
Bashmakov et al.	Resveratrol	54.0(10.1)	6/8	NA	28(3.5)	NA	NA	15(6.9)	3(21)
	Placebo	59.8(6.6)	3/7		29(2.5)			15.2(9.5)	1(10)
Thazhath et al.	Resveratrol	NA	NA	81.1(3.7)	NA	NA	8.1(0.3)	NA	NA
	Placebo			81.1(4.3)			8.2(0.3)		
Timmers et al.	Resveratrol	NA	NA	NA	NA	138(11.8)	7.80(1.62)	NA	NA
	Placebo					141(11.7)	7.70(1.70)		
Javid et al.	Resveratrol	49.1(7.4)	16/5	73.8(10.2)	29.3(4.9)	NA	8.5(3.1)	NA	NA
	Placebo	50.9(8.9)	18/4	70.95(11)	28.3(4.8)		9.4(3.0)		

BMI body mass index, *SBP* systolic blood pressure, *FBG* fasting blood glucose

on the hyperglycemia status in patients with T2DM. Most studies consistently reported reduced glucose concentrations. Movahed et al. [28] showed that 1 g/day of resveratrol supplementation for 45 days markedly reduces fasting blood glucose, insulin, and systolic blood pressure. In addition, Goh et al. [27] provided an important evidence to support that resveratrol is a potential glucose-lowering agent in T2DM patients, either through SIRT1 or AMPK activation. However, Faghihzadeh et al. [33, 34] have reported that supplementation of 500 mg of resveratrol does not have any beneficial effect on fasting blood glucose and insulin resistance markers in patients with non-alcoholic

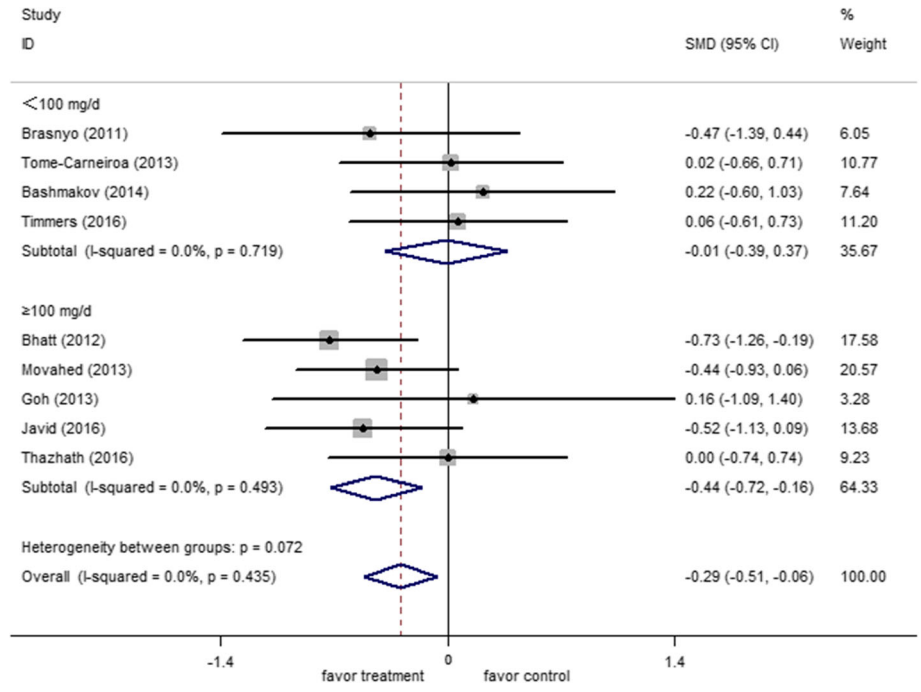

Fig. 2 Forest plot of effect of resveratrol on fasting plasma glucose

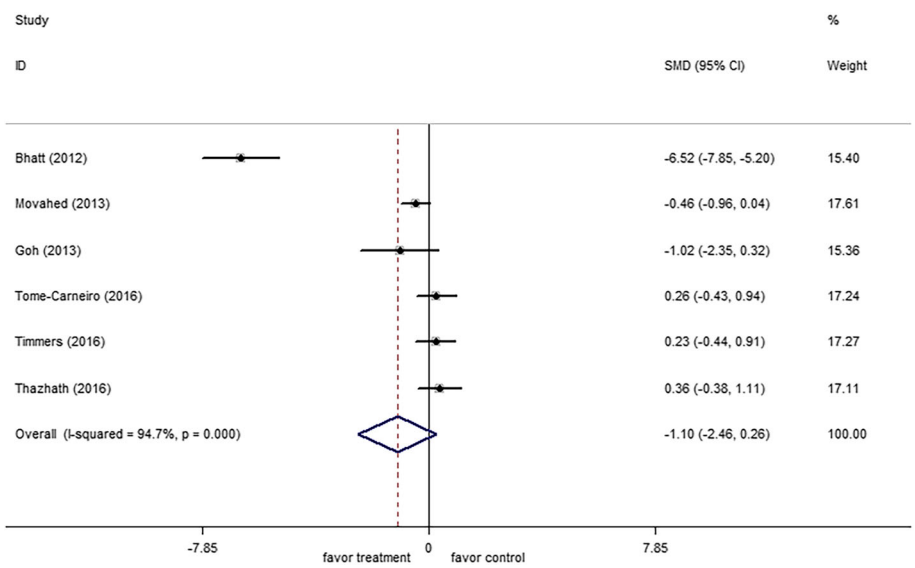

Fig. 3 Forest plot of effect of resveratrol on HbA1c

fatty liver disease. The most possible explanation is that non-diabetic participants have normal baseline glucose levels and insulin concentrations, and resveratrol consumption may not affect the physiological regulation of plasma glucose and insulin in these subjects. Previous study [32] has shown that resveratrol consumption does not significantly affect the plasma measures of glucose control in non-diabetic participants.

The current meta-analysis results revealed that resveratrol supplementation significantly and positively affected the fasting plasma glucose but not the HbA1c. Fasting plasma glucose and HbA1c exhibited different potentially results because fasting glucose reflects only a time point of glucose metabolism. By contrast, HbA1c, which represents the average levels of plasma glucose over a three-month period, is a marker for long-term glucose control. For example, Thazhath et al. [17] reported that 5 weeks of twice daily 500 mg of resveratrol treatment exerts no effect on HbA1c levels in well-controlled diabetic patients. However, Bhatt et al. [14] showed that 3 months of supplementation with 250 mg of resveratrol modestly reduces HbA1c. In the present work, the HbA1c analysis included only five studies with a small number of subjects and three studies with a short follow-up duration (only 5–6 weeks). This duration may be excessively short to reveal any significant

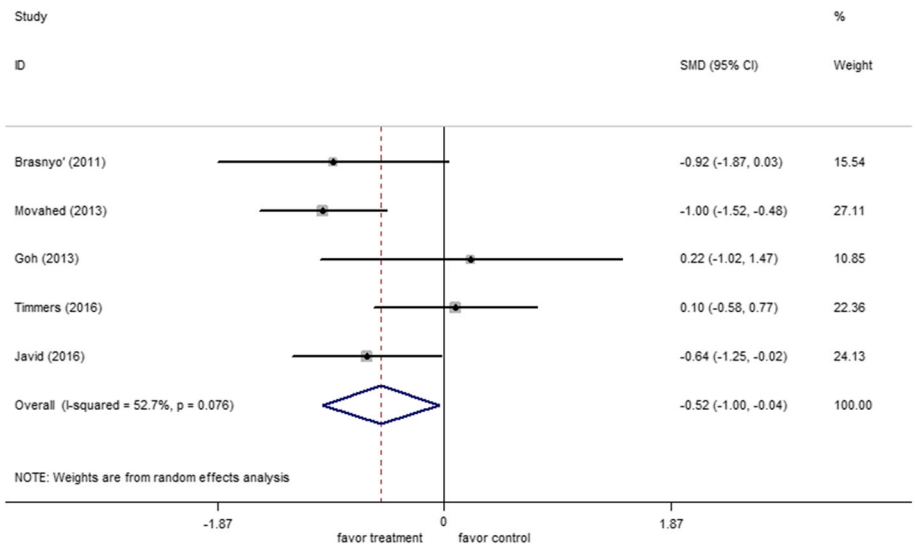

Fig. 4 Forest plot of effect of resveratrol on homeostatic model assessment of insulin resistance

change in the outcome. In addition, a subgroup analysis based on treatment duration could not be performed because of the limited number of studies. Therefore, intervention durations (≥ 12 weeks) might be appropriate for RCTs that assess the effects of resveratrol on glycemic control.

In the present review, the subgroup analyses revealed that high-dose resveratrol (≥ 100 mg/d) supplementation significant reduce the fasting plasma glucose in patients with T2DM. By contrast, the effect of low-dose resveratrol (< 100 mg/d) supplementation was insignificant. These paradoxical results may be attributed to the different resveratrol doses administered in different studies. For instance, the daily administration of resveratrol at doses of 8 [30] and 50 mg [26] did not reveal beneficial effects on metabolic parameter, whereas administration of this drug at higher doses (300, 1000, and 1500 mg) [7, 24, 28] showed beneficial effects on glucose homeostasis. This result suggests a possible direct relationship between the resveratrol dosages and the therapeutic effect. Previous studies indicated that the duration of resveratrol supplementation may influence the outcomes and may differ between preventive and therapeutic clinical studies [6, 32]. Surprisingly, the pooled effects of resveratrol on fasting plasma glucose were not influenced by study duration possibly because of the small sample size.

The HOMA-IR index, a marker of insulin sensitivity, was calculated using fasting insulin and glucose concentrations. Our analysis showed significant reductions in insulin levels and HOMA-IR after resveratrol treatment, thereby suggesting that resveratrol supplementation is beneficial in improving beta cell function and insulin sensitivity and in lowering the insulin levels in patients with T2DM. In accordance with our study, Javid et al. [31] reported a significant decrease in insulin and insulin resistance (HOMA-IR) after resveratrol supplementation for 4 weeks. In *Microcebus murinus*, 33 months of resveratrol treatment improved insulin resistance and glucose tolerance [35]. Experimental and clinical studies suggest that activating inflammatory pathways and oxidative stress may contribute to the pathogenesis of insulin resistance in T2DM. A mechanism that may partly explain the effect of resveratrol on improving insulin sensitivity is the ability of resveratrol to prevent inflammation by improving cellular stress and inhibiting inflammatory gene expression [9, 36]. Resveratrol reduces insulin secretion and thus decreases ATP content and protects the diabetic pancreas from hyperglycemia.

Type 2 diabetics with hypertension and non-high density lipoprotein cholesterol have higher probability to contract atherosclerotic cardiovascular diseases than those with normal variables [37]. The current meta-analysis revealed that the resveratrol supplementation significantly reduced systolic blood pressure and diastolic blood pressure, although such topic has not been systematically studied in hypertensive subjects. The drop in blood pressure has been observed with resveratrol. Timmers et al. [29] reported that 30 days of 150 mg/d resveratrol supplementation reduces systolic blood pressure levels. Furthermore, Bhatt et al. [14] showed the same effect on patients with T2DM. On one hand, resveratrol acutely improves vascular endothelial function and blood flow in humans. On the other hand, small but significant reductions in blood pressure may be attributed to the improvements in glucose homeostasis and insulin resistance. In the current work, we found no significant change in the serum lipid profiles of T2DM patients. Studies in humans and animal models suggest that resveratrol exert beneficial effects on lipids by modulating the genes involved in lipid metabolism [38, 39]. However, an [28] included study reported a positive effect for HDL. All other studies reported insignificant effects for increased HDL levels. Decreasing trends were also observed, but no significant change was noted in the LDL levels after resveratrol supplementation. The effects of resveratrol on lipids were apparent only in obese subjects and not in subjects with low body mass index. Resveratrol could have failed to influence metabolism because most patients were not obese.

Our review features a number of strengths. This review includes all available RCTs addressing the clinical question and is the most up-to-date systematic review of the topic. Our review also considered the dose and duration of resveratrol supplementation in T2DM patients in great detail. Nevertheless, several limitations exist in the present analysis. First, the study used the data provided by the published literature, and the data for each patient were unavailable. Hence, test condition bias might exist. Second, some included studies were of low quality because random allocation schemes were not hidden. Thus, the findings were unreliable. With reduced reliability, the results should be treated with caution in clinical practice. Third, the sample size of the included RCTs was so small that significant metabolic changes associated with resveratrol might not have been detected. Another limitation is that the forms of resveratrol obviously differed. Extracts and powders may provide different bioactive compounds with varying levels of potency and bioavailability. The differences in dose and duration might have also affected the accuracy of the results. Therefore, high-quality studies are required to determine the dose-dependent effects at varying treatment periods.

Conclusions

This study provides novel insights into the beneficial effects of resveratrol supplementation on T2DM. Specifically,

resveratrol supplementation may improve fasting plasma glucose, HOMA-IR, and insulin in diabetic patients. This result proves that the drug may ameliorate metabolic parameters. The dose and/or duration of treatment with resveratrol might also influence the effect of resveratrol on glucose homeostasis. Therefore, studies with durations longer than three months should be designed to confirm the efficacy of resveratrol and determine the appropriate dosage regimen in managing T2DM. Most of the included articles did not explain whether adverse events occurred in the studies. The long-term risks and benefits of resveratrol supplementation are unknown. Understanding the efficacy of resveratrol in diabetic patients requires large-scale, well-designed, and population-based studies in the future.

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