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RESEARCH ARTICLE

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The effects of resveratrol supplementation on *PPARα*, *p16*, *p53*, *p21* gene expressions, and sCD163/sTWEAK ratio in patients with type 2 diabetes mellitus: A double-blind controlled randomized trial

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The present study sought to evaluate the effect of resveratrol supplementation on mRNA expression levels of peroxisome proliferator-activated receptor alpha (*PPARα*), *p53*, *p21*, *p16*, and serum levels of cluster of differentiation 163 (CD163) to TNF-like weak inducer of apoptosis (TWEAK) ratio in patients with type 2 diabetes. In this double-blind randomized controlled trial, 71 patients were randomly assigned to receive either 1,000 mg of trans-resveratrol or placebo (methyl cellulose) for 8 weeks. Expression levels of genes of interest, and serum levels of sCD163 and sTWEAK were assessed at baseline and at the end of the study. Resveratrol supplementation significantly increased mRNA expression levels of *p53* and *p21* genes, compared with the placebo group (fold change of *p53* = 1.29, p = .04; fold change of *p21* = 1.46, p = .006). However, no significant effect on expression levels of *PPARα* and *p16* genes was observed after supplementation. In addition, resveratrol significantly reduced serum levels of sCD163/sTWEAK ratio compared with the placebo group (p = .003). Resveratrol supplementation resulted in significant changes in *p53* and *p21* genes expression, while serum levels of sCD163/sTWEAK ratio also improved in the resveratrol group, without any significant change in adjusted sCD163 levels. More research is needed to confirm the beneficial effects of resveratrol for patients with diabetes.

KEYWORDS
cardiovascular diseases, resveratrol, sCD163, sTWEAK, type 2 diabetes mellitus

1 | INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a major cause of morbidity and mortality, globally (Chen, Magliano, & Zimmet, 2012; Fowler, 2007; Whiting, Guariguata, Weil, & Shaw, 2011), and related hyperglycemia can result in widespread macro- and micro-vascular complications, as well as atherosclerosis (Toupchian et al., 2018). The incidence of intimal hyperplasia (IH), following atherosclerotic lesions formation, is prevalent in T2DM, and is associated with cardiovascular events; the most common cause of mortality in patients with diabetes (Fowler, 2008; Polak et al., 2011; Toupchian, Sotoudeh, Mansoori, Nasli-Esfahani, et al., 2016). Recent studies have indicated that the proliferation of vascular smooth muscle cells (VSMCs), along with other underlying factors, is linked to IH (Gizard et al., 2005; Wright et al., 2011).

There are swathes of evidence suggesting that one of the nuclear receptors superfamily, named peroxisome proliferator-activated receptors (PPARs), can modulate the proliferation of VSMCs (Gizard et al., 2005, 2008). PPARα—as a member of this family—has critical roles in fatty acid oxidation, glucose metabolism, vascular function, plaque stability, and cell proliferation (Chinetti-Gbaguidi, Fruchart, & Staels, 2005; Toupchian et al., 2016). In previous animal (Gizard et al., 2005, 2008) and human studies (Toupchian et al., 2016; Toupchian et al., 2018), the role of activated PPARα in the inhibition of VSMCs proliferation has been proposed through the tumor suppressor *p16* gene. P16, or cyclin-dependent kinase inhibitor 2A, can bind and inhibit the action of D-type cyclin-dependent kinases (CDK4 and CDK6) and arrest the cell cycle in the transition from G_1 to S phase (Sherr, Beach, & Shapiro, 2016).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a phytoalexin polyphenol found abundantly in red grapes and nuts (Storniolo & Moreno, 2018), has been introduced as a natural exogenous ligand for PPARα, and it appears capable of suppressing cell cycle progression through the p16 pathway (Inoue et al., 2003; Qin, Lu, & Rodrigues, 2014; Takizawa et al., 2015). Moreover, some studies have posited that this bioactive compound has a putative role in phosphorylation and activation of p53 (Dong, 2003; She, Bode, Ma, Chen, & Dong, 2001). P53 is a tumor suppressor and anti-cancer agent that upregulates the cyclin-dependent kinase inhibitor 1 (*p21*) gene, which results in the cell-cycle arrest at the S/G_2 phase (Cayrol, Knibiehler, & Ducommun, 1998; Coppé et al., 2008). Some studies have addressed the potential role of p53 in the reduction of intimal thickness in animal models (Chang, Barr, Lu, Barton, & Leiden, 1995; Guevara, Kim, Antonova, & Chan, 1999; Rosso et al., 2006; Tanner et al., 1998).

Chronic inflammation caused by macrophage hyperactivity and overexpression of pro-inflammatory cytokines occurs in diabetes, and especially in obese patients (Kern, Ranganathan, Li, Wood, & Ranganathan, 2001). The cluster of differentiation 163 (CD163) is a macrophage scavenger receptor, which is involved in iron recycling and has anti-inflammatory effects (Moestrup & Møller, 2004); indeed, CD163 has been acknowledged as a neutralizing receptor for TNF-like weak inducer of apoptosis (TWEAK), a proinflammatory cytokine that mediates its effects through the Fn14 receptor (Moreno et al., 2009). Moreover, it has recently been proposed that serum levels of CD163 to TWEAK ratio (sCD163/sTWEAK) can be used as an indicator of the vascular disease severity (Llaurado et al., 2012; Moreno et al., 2010; Urbonaviciene et al., 2011).

Despite existing evidence regarding the beneficial effects of resveratrol on cardiovascular diseases, to our knowledge, there is no study that has investigated the effect of this antioxidant on intimal hyperplasia through the proposed mechanisms. Thus, we sought to perform a randomized clinical trial to determine the effect of resveratrol supplementation on the gene expression of *PPARα*, *p16*, *p53*, *p21*, and the serum levels of sCD163/sTWEAK ratio, in patients with T2DM.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

This research was an 8-week, double-blind, randomized controlled trial. This study was conducted in complete agreement with the Helsinki declaration, and the protocol was approved by the Medical Ethics Committee of Yazd University of Medical Sciences and registered in the Iranian Registry of Clinical Trials (www.irct.ir: IRCT20171118037528N1).

Male and female volunteers, aged between 30 and 60 years, who had been diagnosed with T2DM, and with body mass index (BMI) between 25 and 30 (kg m^{-2}), were eligible to participate in the study. Patients were excluded if they (a) were pregnant or lactating, (b) had a history of any type of cancer, renal or liver failure, gastrointestinal ulcers, Alzheimer's disease and, cardiovascular complications (c) had glycated hemoglobin above 8% or were receiving insulin treatment, (d) consumed any antioxidant supplements (for example, vitamin C, E, A or fish oil supplements), anticoagulants, fibrate lipid-lowering agents, and anti-inflammatory drugs or drank alcoholic beverages (for at least 6 months before the study).

Eligible subjects were invited and, after providing further information, a consent form was signed by willing patients. Stratified block randomization, based on sex and age (30–45 and 45–60 years), using computer-generated random numbers, was performed by an independent statistician to allocate participants into intervention and control groups (in a 1:1 allocation ratio).

Given that it has been reported that 1,000 mg/day of resveratrol is well tolerated and shows no toxic effects in patients with diabetes (Movahed et al., 2013; Thazhath et al., 2016), participants were instructed to take 1000 mg/day (two 500 mg capsules) micronized trans-resveratrol (Mega-Resveratrol, Danbury) and methylcellulose (Barij essence, Kashan, Iran), in intervention and control groups, respectively, for an 8-week treatment period. Each capsule of resveratrol supplement provided 500 mg of 99.71% micronized trans-resveratrol, with particle sizes lower than 1.9 μm, and free from inactive ingredients, fillers, flavoring agents, and additives. Resveratrol and placebo capsules were completely identical in shape, color, and taste, and packed in the same bottles. Filling and labeling of the containers as

A and B was performed by a person who was not involved in the process of the study, and the content of the bottles remained unknown to the participants and researchers. Patients were asked to bring back the containers with remaining capsules at the end of the first month to calculate compliance. More information about the study methodology has been published previously (Abdollahi, Salehi-Abargouei, Tabatabaie, et al., 2019).

Weight and body composition were measured at the beginning and at end of the study, in an overnight fasted state, using a body analyzer machine (Tanita BC-418, Tokyo, Japan). Height, waist, and hip circumferences were measured according to the standard protocol (Kamal, 2006). Body mass index (BMI) was calculated mathematically, as weight (in kg), divided by height in m^2. All participants were asked not to change their dietary habits or physical activity during the study period. However, a physical activity questionnaire (MET; Aadahl & Jørgensen, 2003) and 3-day food records were obtained at week 0 and week 8 of the study. Data from diet records were analyzed using Nutritionist IV software and converted into macronutrient and micronutrient intakes. Dietary total flavonoid also was calculated using the USDA Database for the Flavonoid Content of Selected Foods (Bhagwat, Haytowitz, & Holden, 2014). Dietary intake, physical activity, as well as anthropometric data have been reported previously (Abdollahi, Salehi-Abargouei, Toupchian, et al., 2019).

2.2 | Biochemical measures

After overnight fasting, a venous blood sample (10 ml) was collected at the beginning and at the end of the study for biochemical and gene expression assessments. Aliquots of serum samples were stored in −70°C after centrifugation (3,000*g*, 10 min at room temperature; Eppendorf AG, Hamburg). Serum levels of sCD163 and sTWEAK were assessed by enzyme-linked immunoassay (ELISA) method according to the manufacturer protocol (ZellBio, Germany; inter and intra-assay coefficient of variations were <12% and <10%, respectively, for both sCD163 and sTWEAK). For gene expressions assessment, total RNA was extracted directly from whole blood using GeneAll Hybrid-R RNA purification kit (GeneAll Biotechnology Co., Seoul, South Korea). After checking the quality and purity (260/280 nm ratio between 1.8 and 2.2) of the RNA (NanoDrop, Thermo Scientific), total mRNA was reverse-transcribed to the first-strand cDNA by cDNA synthesis kit (GeneAll Biotechnology Co., Seoul, South Korea). The cDNA was amplified by real-time polymerase chain reaction (RT-PCR), and SYBR Green method (Takara Bio, Inc., Japan) to determine the gene expression levels of *PPARα*, *p53*, *p21*, and *p16* (Applied Biosystems). Glyceraldehyde phosphate dehydrogenase (GAPDH) was considered as a housekeeping gene in all assessments. Three primer designing tools (Primer Blast, Oligocalc, and Gene runner 5.0.99) were applied for sequencing the study primers (Table 1). PCR efficacy and changes in the expression levels were tested using LinRegPCR software (Robledo et al., 2014) and Pfaffl equation (Pfaffl, 2001), respectively.

2.3 | Sample size

As, at the time of the study design, there were no human studies on the effect of resveratrol on the expression of interested genes, the sample size was calculated based on a previous study investigating the PPARα expression in peripheral blood mononuclear cells (PBMCs; D'Amore et al., 2013). Considering $\alpha = .05$, power of 80%, and a 20% drop-out rate, the final sample size was set to be 36 participants in each group. However, a power analysis was conducted to assess quantity of sample size for outcomes reported in the current manuscript. Furthermore, sample size was recalculated based on values reported for total cholesterol (our secondary outcome) on a published study (Bhatt, Thomas, & Nanjan, 2012), and final sample size was determined to be 40 subjects for each group. Therefore, our sample size provides enough power to conduct the analyses.

2.4 | Statistical analysis

Data entry and statistical analyses were performed using SPSS for Windows (SPSS, Chicago, IL), version 23.0. Data were presented as proportions or mean ± *SD* for categorical or continuous data, respectively. A one-sample Kolmogorov–Smirnov test was conducted on each variable to test the normality distribution of the data. Independent samples and paired samples *t* tests were carried out for comparison of continuous data between and within the study groups, respectively. *Analysis of covariance* (ANCOVA) was applied to adjust the possible confounders (age, gender, and baseline BMI) when assessing between-group differences. Last observation carried forward imputation was applied to manage missing data (Little et al., 2012). Statistical significance was accepted at $p \leq .05$, for all comparisons.

TABLE 1 Real-time PCR primer sequences

	Forward	Reverse
p53	5′GAGCTGAATGAGGCCTTGGA3′	5′CTGAGTCAGGCCCTTCTGTCTT3′
p21	5′TGGAGACTCTCAGGGTCGAAA3′	5′GGCGTTTGGAGTGGTAGAAATC3′
p16	5′CTTCCTGGACACGCTGGTG3′	5′GCATGGTTACTGCCTCTGGTG3′
PPARα	5′CTATCATTTGCTGTGGAGATCG3′	5′AAGATATCGTCCGGGTGGTT3′
*GAPDH**	5′TGGTATCGTGGAAGGACTCATG3′	5′GCTTCACCACCTTCTTGATGTC3′

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; PPARα, peroxisome proliferator activated receptor alpha.

3 | RESULTS

A total of 76 patients were enrolled in this 8-week intervention study. Five of the participants (three patients in resveratrol group and two patients in the placebo group) dropped out because of disinclination to continue participating in the study (n = 3), pregnancy (n = 1), and traveling (n = 1). Finally, 71 participants (35 patients in the resveratrol group and 36 patients in the placebo group) completed the study (Figure 1). No significant study-related adverse event was observed; although, two patients reported tolerable gastrointestinal distress following resveratrol supplementation. The compliance rate of participants was calculated as 93.1% in the resveratrol group and 92.6% in the placebo group, based on the residual capsules.

Baseline characteristics of the participants are summarized in Table 2. Lifestyle factors, including dietary intake, total flavonoid, and physical activity, remained unchanged during the study (data not shown).

The effect of the intervention on relative changes in mRNA expression levels of *p16*, *p21*, *p53*, and *PPARα* is detailed in Figure 2. The analyses revealed that there was a 1.29-fold increase in *p53* gene expression levels (p = .04), and 1.46-fold increase in gene expression levels of *p21* (p = .006), in the resveratrol group compared with the placebo group. However, there were no significant differences between the two groups in the expression of *p16* and *PPARα* genes.

Furthermore, in comparison with placebo, resveratrol supplementation yielded a significant reduction in sCD163 levels in the unadjusted model (−176.85 ± 310.7 ng/ml, p = .04). However, no significant change was observed after adjusting for age, gender, and baseline BMI. We also found a significant increment in sTWEAK levels, in both unadjusted and adjusted models, compared with the control group (394.2 ± 903.9 pg/ml, p < .05). Moreover, comparing the mean changes in sCD163/sTWEAK ratio highlighted significant differences between the two groups (−0.09 ± 0.13, p = .001); which remained unchanged after controlling for possible confounders (p = .003; Table 3). The results remained stable when intention-to-treat analysis was applied (Tables S2 and S3).

4 | DISCUSSION

The results of this study showed that 8 weeks resveratrol administration, in patients with T2DM, upregulated mRNA expression of *p53* and *p21* genes, without any significant effect on the expression of *PPARα* and *p16* genes. In addition, serum levels of sCD163 to

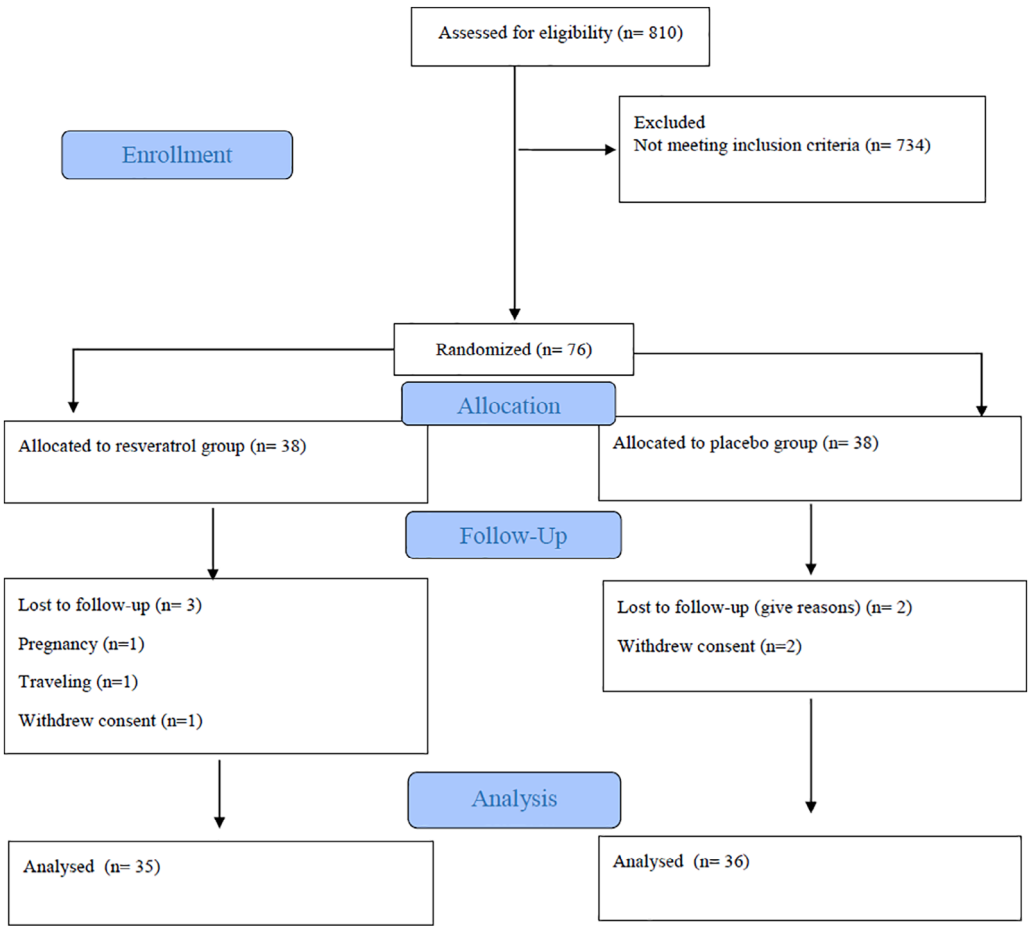

FIGURE 1 CONSORT diagram outlining the number of subjects involved in enrollment, intervention allocation, follow-up, and data analysis [Colour figure can be viewed at wileyonlinelibrary.com]

sTWEAK ratio significantly decreased as a result of resveratrol supplementation.

To our knowledge, no study has investigated the effect of resveratrol on gene expression of *PPARα*, *p16*, *p53*, *p21*, and the serum levels of sCD163/sTWEAK ratio, in patients with T2DM. However, there are some cell line studies in this regard; for instance, Zakar et al., in a study on VSMCs, showed that resveratrol can stimulate the cellular signaling of p53 in both the nucleus and cytoplasm (Mnjoyan & Fujise, 2003). Another study suggested that resveratrol activates p53 in a dose-dependent manner (Howitz et al., 2003), and it has been proposed that acetylation and phosphorylation at the serine-15 residue of p53 by resveratrol can increase the activity and stability of this tumor suppressor (She et al., 2001). Resveratrol has also been shown to inhibit p53 deacetylation, which is mediated by NAD-dependent deacetylase sirtuin-1 (SIRT1) (Howitz et al., 2003). Similarly, this phenol can increase p21 levels in a dose-dependent manner, and seems to be related to elevated p53 and its signaling pathways (Mnjoyan & Fujise, 2003). There is some evidence supporting the effect of resveratrol in p53 activation and p21—as its target gene—in cancerous cells (Lu, Ho, Ghai, & Chen, 2001; Shih, Davis, Lin, & Davis, 2002), but the present study is the first to propose the same changes in PBMC of patients with diabetes.

TABLE 2 Baseline characteristics of the study participants

Variables[a]	Resveratrol (*n* = 35)	Placebo (*n* = 36)
Age (years)	50.14 ± 7.38	50.06 ± 7.69
Diabetes duration (years)	9.40 ± 7.07	8.11 ± 6.90
Gender (female), *n* (%)	15 (42.9)	16 (44.4)
Height (cm)	164.94 ± 7.22	162.08 ± 11.29
Weight (kg)	73.69 ± 8.24	72.71 ± 10.52
BMI (kg m^{-2})	27.10 ± 2.69	27.66 ± 2.71
Smoker, *n* (%)	5 (14.3)	2 (5.6)
Complications		
Hypertension, *n* (%)	11 (31.4)	7 (19.4)
Kidney disorders, *n* (%)	2 (5.7)	3 (8.3)
Hepatic disorders, *n* (%)	3 (8.6)	2 (5.6)
Neuropathy, *n* (%)	2 (5.7)	2 (5.6)
Retinopathy, *n* (%)	5 (14.3)	5 (13.9)
Family T2DM history, *n* (%)	25 (71.4)	30 (83.3)
Medications		
Metformin, *n* (%)	30 (85.7)	31 (86.1)
Glibenclamide, *n* (%)	*11 (31.4)*	*16 (44.4)*
Statins, *n* (%)	3 (8.6)	4 (11.1)
Blood pressure lowering drugs, *n* (%)	6 (17.1)	5 (13.9)

Abbreviations: BMI, body mass index; T2DM, type 2 diabetes mellitus.
[a]Data are expressed as mean ± *SD* for continuous variables or as frequency (percentage) for categorical variables.

In our study, mRNA genes expression level of *PPARα* or *p16* did not change following resveratrol supplementation. It has been reported that resveratrol can bind to the PPARs and stimulate their transcription activities (Inoue et al., 2003), although the activation of PPARs by resveratrol has been observed in in-vitro studies, the results are inconsistent across different cell lines, based on the presence or absence of coactivators or corepressors (Calleri et al., 2014). It seems that tissue distribution of PPARα is an important factor that should be considered, while it should also be acknowledged that the *PPARα* gene is expressed at a low level in PBMC, and this can explain our findings. Consequently, the theory of upregulation of p16 by PPARα activation, through binding to the PPAR response element in p16 promoter (Florence Gizard et al., 2005), was not approved by our results.

TWEAK belongs to the TNF superfamily, is generated mainly from macrophages, and is released into the bloodstream in its functional form (sTWEAK; Chicheportiche et al., 1997). Studies have reported unexpected reductions in sTWEAK in inflammatory diseases, such as T2DM (Jelic-Ivanovic et al., 2009; Kralisch et al., 2008), and although the exact cause of the reduction is not clear, some mechanisms related to the CD163 and Fn14 receptor have been suggested (Moreno et al., 2009; Urbonaviciene et al., 2011).

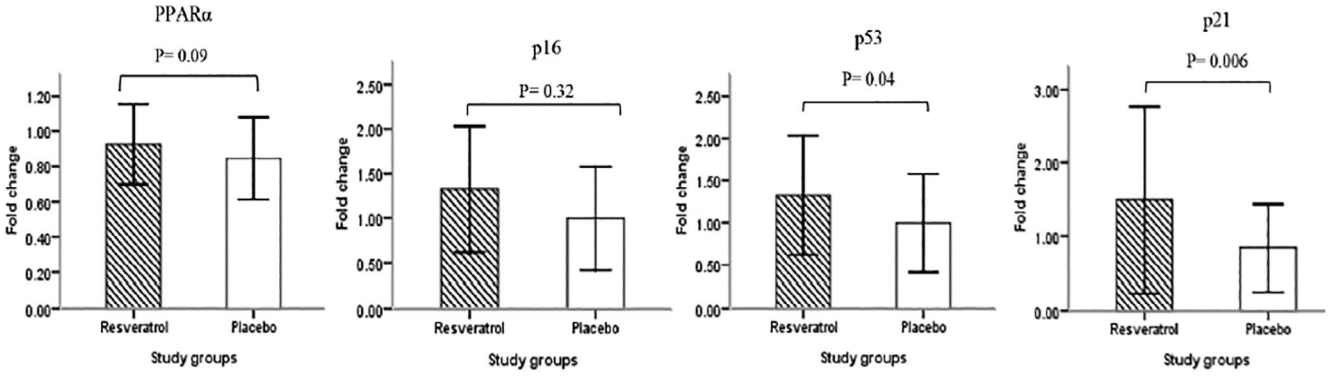

FIGURE 2 Fold changes (means ± *SDs*) in gene expression levels of *PPARα*, *p16*, *p53*, and *p21* in patients with Type 2 diabetes mellitus receiving resveratrol (*n* = 35) or placebo (*n* = 36). The presented *p* values are associated with fold changes comparisons adjusted for age, gender, and baseline body mass index obtained from analysis of covariance (ANCOVA)

TABLE 3 Serum levels of CD163 and TWEAK values during study in resveratrol and placebo groups[a]

	Resveratrol (n = 35)				Placebo (n = 36)					
	Before	After	Change	p value[b]	Before	After	Change	p value[b]	p value[c]	p value[d]	Power
sCD163 (ng/ml)	1,353.43 ± 339.2	1,176.57 ± 282.4	−176.85 ± 310.7	.002	1,100 ± 383.14	1,086 ± 289	−18.8 ± 343.3	.74	.04	.8	0.84
sTWEAK (pg/ml)	3,343.14 ± 918.3	3,737.4 ± 1,193.4	394.2 ± 903.9	.01	3,648 ± 1,017.5	3,308.5 ± 1,173.6	−339.4 ± 1,123	.08	.004	.001	0.8
sCD163/sTWEAK	0.43 ± 0.15	0.34 ± 0.11	−0.09 ± 0.13	<.001	0.33 ± 0.19	0.36 ± 0.15	0.25 ± 0.14	.29	.001	.003	0.81

Abbreviations: sCD163, Serum level of cluster of differentiation 163; sTWEAK, Serum level of TNF-like weak inducer of apoptosis.

[a]All variables are expressed as mean ± SD.

[b]The presented p values are associated with before and after intervention comparisons obtained from paired t test.

[c]The presented p values are associated with mean changes comparisons obtained from independent samples t test.

[d]The presented p values are associated with mean changes comparisons adjusted for age, gender, and baseline BMI obtained from analysis of covariance (ANCOVA).

Indeed, studies have shown that both serum and mRNA expression levels of CD163 are higher in inflammatory conditions, such as T2DM, obesity, or atherosclerosis (Kawarabayashi et al., 2017). CD163 is a scavenger receptor located on macrophages and involved in the hemoglobin–haptoglobin complexes removal, and is also known as a scavenger of sTWEAK (Bover et al., 2007; Fabriek, Dijkstra, & van den Berg, 2005). Resveratrol is an antioxidant and can activate a variety of antioxidant enzymes, such as catalase, glutathione peroxidase, glutathione transferase, and superoxide dismutase, which can elicit a consequential reduction of monocyte/macrophage activity and CD163 (Inglés et al., 2014; Toupchian, Sotoudeh, Mansoori, Nasli-Esfahani, et al., 2016). Therefore, sTWEAK levels are increased following CD163 reduction. Resveratrol also upregulates expression levels of SIRT1 and 5′ AMP-activated protein kinase (AMPK), which have inhibitory interactions with NF-κB (Xu, Botchway, Zhang, Zhou, & Liu, 2018), and, as a result, the TWEAK-Fn14 pathway is suppressed, which can lead to increases in sTWEAK levels. However, significant improvement in sCD163 disappeared after adjustment for age, gender, and baseline BMI, in our results. It has been reported that higher white adipose tissue is associated with higher sCD163 concentration, representing obesity-induced inflammation, and macrophage activity (Kračmerová et al., 2014). In accordance with previous study, visceral adipose tissue is an important predictor of sCD163 concentration in patients with type 2 diabetes (Sørensen et al., 2015). As visceral adipose tissue is greater in female than male, sex differences also may confound results (Karastergiou, Smith, Greenberg, & Fried, 2012).

The present study is the first human trial to investigate the effects of resveratrol supplementation on the cellular factors associated with intimal hyperplasia and the first randomized controlled trial that used micronized resveratrol supplement as a natural ligand for PPARα. However, a few limitations need to be considered. One of the limitations of our study was the relatively short-term intervention, which did not allow investigators to discern the long-term effects of resveratrol on the studied variables. Moreover, in this study, we assessed the mRNA expression levels of PPARα, however, it seems PPARα activity may be more important for interpreting results. Another limitation in this study was the surrogate markers that were used for endothelial function assessment, instead of gold-standard methods, such as flow-mediated dilation (FMD) or peripheral arterial tonometry (PAT).

5 | CONCLUSIONS

We found that 8 weeks supplementation with micronized resveratrol, in patients with T2DM, improved sCD163/sTWEAK ratio; despite no significant change in adjusted sCD163 levels, *p53* and *p21* gene expressions were upregulated. However, there is a need for further long-term trials to confirm the veracity of these results.

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CONFLICT OF INTEREST

The funding bodies had no role in the design of the study, writing, or decision to submit the manuscript for publication. They also had no role in any aspect of the described data management, analysis, or the reporting of results. The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Shima Abdollahi, Amin Salehi-Abargouei, Omid Toupchian, Javad Heshmati, and Hassan Mozaffari-Khosravi: Were involved in initial idea of this study and designing the trial. **Omid Toupchian, Shima Abdollahi, Amin Salehi-Abargouei, Cain C. T. Clark, and Hassan Mozaffari-Khosravi:** Contributed to writing the manuscript and securing the grant. **Omid Toupchian and Javad Heshmati:** Were co-investigators and involved in collecting data, concealment procedure, and counseling patients. **Hossein Fallahzadeh**: Provided statistical expertise in clinical trial design, sample size calculation, and blinding. **Mohammad Hasan Sheikhha:** Contributed to the design of biochemical procedures. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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