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# Resveratrol supplementation decreases blood glucose without changing the circulating $CD14^+CD16^+$ monocytes and inflammatory cytokines in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled study

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Running Title: Resveratrol had no effect on inflammation

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**Abbreviations**

AP-1: activator protein-1, AMPK: adenosine monophosphate-activated protein kinase, BMI: body mass index, $CD14^{+}CD16^{+}$: cluster of differentiation 14,16, DBP: diastolic blood pressure, FBS: Fasting blood glucose, HDL-C: high density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, HOMA-β: homeostasis model assessment of β-cell function, hsCRP: high sensitivity C-reactive protein, IL: Interleukin, LDL-C, low density lipoprotein cholesterol, LPS: Lipopolysaccharide, MCP-1: monocyte chemoattractant protein-1, NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, PBMCs: Peripheral blood mononuclear cells, SBP: systolic blood pressure, SGOT: serum glutamate oxaloacetate transaminase, SGPT: serum glutamate pyruvate transaminase, TG: triacylglycerol, TLR: toll-like receptor, T2D: Type 2 diabetes, TNF-α: Tumor necrosis factor alpha, WC: Waist circumference, W/H: waist to hip ratio

**Abstract**

Chronic low-grade inflammation is the hallmark of type 2 diabetes (T2D). Although in vitro and animal studies have shown that resveratrol exerts anti-inflammatory effects, clinical trials addressing these effects in patients with T2D are limited. Therefore, in the present study, we hypothesized that supplementation of resveratrol might improve inflammatory markers in patients with T2D in a randomized double-blind placebo-controlled clinical trial. A total of 45 T2D patients were supplemented with either of 800 mg/day resveratrol or placebo capsules for eight weeks. Percentage of $CD14^{+}CD16^{+}$ monocytes, plasma levels of inflammatory cytokines (TNF-α, IL-1β, IL-6 and MCP-1), the expression levels of genes involved in the inflammatory responses (TLR-2, TLR-4 and NF-κB), lipopolysaccharide (LPS)-stimulated cytokine (TNF-α, IL-1β, and IL-6) secretion from PBMCs, as well as metabolic and anthropometric parameters, were assessed at both the baseline level and the end of the study. Compared with the placebo group, we could not detect any significant difference in the percentage of $CD14^{+}CD16^{+}$ monocytes, LPS-induced cytokine secretion, plasma levels of inflammatory cytokines, and the expression of inflammatory genes in resveratrol group. Moreover, we did not find any significant change in the metabolic and anthropometric parameters except for a significant reduction in fasting blood glucose and blood pressure. In conclusion, eight weeks supplementation of resveratrol reduces blood glucose level in patients with T2D without improving their inflammatory markers.

**Keywords**: Inflammation, Type 2 diabetes, $CD14^{+}CD16^{+}$ Monocytes, Resveratrol, PBMC, Clinical trial

## 1. Introduction

Type 2 diabetes (T2D) is one of the most serious health problems in the world. According to a World Health Organization report, 347 million people worldwide suffered from T2D in 2008 and this number is estimated to be doubled by 2030 [1].

Emerging evidence suggests that chronic low-grade inflammation has an important role in the pathogenesis of T2D [2]. Chronic inflammation is characterized by increased abundance of macrophages and enhanced production of inflammatory cytokines in different metabolic tissues [3]. Monocytes and monocyte-derived macrophages are suggested to be a major source of local and systemic inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and IL-6 [2]. Peripheral blood monocytes are a heterogeneous population that are mainly divided into two distinct subpopulations based on the expression of CD14 and CD16. The major monocyte subset, accounting for approximately 90% of the total monocyte population, expresses high levels of CD14 and no CD16 ($CD14^{++}CD16^{-}$), while $CD14^{+}CD16^{+}$ monocytes that constitute a small percentage of total monocyte population express CD16 and low level of CD14. The $CD14^{++}CD16^{-}$ monocytes seem to be anti-inflammatory, as they produce the cytokine IL-10 in response to bacterial lipopolysaccharide (LPS). Conversely, the $CD14^{+}CD16^{+}$ monocytes seem to be pro-inflammatory, because they produce pro-inflammatory mediators in response to LPS [4, 5]. The percentage of $CD14^{+}CD16^{+}$ monocytes is increased in various inflammatory diseases including end-stage renal disease, coronary artery disease, and T2D [6-8]. Inflammatory monocytes exert their crucial role in inflammatory pathways through toll-like receptors (TLRs). Particularly, the expression of TLR2 and TLR4 has been shown to increase in T2D patients [9]. Activation of TLR2 and TLR4 results in the production of TNF-α, IL-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1) [2, 9, 10]. These cytokines can induce insulin

resistance and β cell dysfunction in various tissues. Therefore, strategies to restrain chronic inflammation may be effective in treating many chronic diseases including T2D. In this context, natural products are documented to reduce systemic low-grade inflammation and are acknowledged as the anti-inflammatory interventions.

Resveratrol (trans-3,5,4'-trihydroxystilbene), a naturally occurring polyphenolic compound, which is found in grapes, berries, and peanuts has been shown to prevent and treat chronic conditions including cardiovascular disease and neurodegenerative disorders [11-13]. The anti-diabetic properties of resveratrol have also been extensively studied in recent years. Meta-analysis of 11 randomized controlled trials demonstrated that resveratrol significantly improves glucose control and insulin sensitivity in patients with diabetes [14]. The evidence has demonstrated that the beneficial effects of resveratrol on health are mediated through its antioxidant, cardioprotective, and neuroprotective activities [13]. Studies using cultured cells, and laboratory animals have also suggested that resveratrol has anti-inflammatory property [15]. Resveratrol downregulates the inflammatory response through inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, and inhibition of activated immune cells via its inhibitory effects on nuclear factor κB (NF-κB) and the activator protein-1 (AP-1) [16]. Furthermore, randomized clinical trial studies have provided the evidence that resveratrol might reduce inflammatory markers in different clinical conditions [17, 18]. Although most studies have shown an anti-inflammatory effect of resveratrol in human subjects, limited information is available about the resveratrol supplementation effects on inflammation in T2D patients. Therefore, in the present study, we hypothesized that supplementation of resveratrol might improve the inflammatory markers in patients with T2D. To test the hypothesis, we measured the percentage of circulating $CD14^{+}CD16^{+}$ monocytes, plasma levels of inflammatory

cytokines (TNF-α, IL-1β, IL-6 and MCP-1), the expression levels of genes involved in the inflammatory responses (TLR-2, TLR-4 and NF-κB), lipopolysaccharide (LPS)-stimulated cytokine (TNF-α, IL-1β, and IL-6) secretion from PBMCs as well as metabolic and anthropometric parameters in patients with T2D in a randomized, double-blind, placebo-controlled clinical trial.

## 2. Methods and materials

### 2.1. Study design and participants

A total of 45 patients with T2D who repeatedly visited the Endocrine & Metabolism Research Institute- Tehran University of Medical Sciences, Tehran, Iran, participated in this randomized, double-blind, placebo-controlled clinical trial.

Inclusion criteria were: being diagnosed with T2D, HbA1c ≥7, age between 30 and 70 years, body mass index (BMI) <35 kg/m$^2$, being on a diet and/or hypoglycemic agents other than insulin, having no allergy to grapes, green tea, and peanuts, and willingness to give written informed consent.

Exclusion criteria were: having type 1 diabetes, being pregnant, being lactating mothers, treatment with antioxidant substance, treatment with insulin, anticoagulants, steroids, or anti-inflammatory drugs, uncompensated diabetes, being with severe heart disease, having hepatic disease or kidney diseases, presence of diabetes-related chronic complications, any severe chronic or life-threatening diseases, unstable body weight (weight gain or loss >3 kg in the previous 3 months), and allergy to grapes, green tea, and peanuts. The patients were randomly assigned to resveratrol and placebo groups.

### 2.2. Informed consent and ethics committee approval

The study was approved by the Ethics Committee of Tehran University of Medical Sciences. The trial was also registered in the Iranian Registry of Clinical Trials (IRCT) under the registration number IRCT2015080223336N2. The informed written consent was obtained from each patient.

### 2.3. Randomization

Stratified randomization was used to ensure that equal numbers of male and female are allocated to each group. Randomization of the participants into placebo and resveratrol groups was then performed by a computer-generated random number list.

### 2.4. Blinding

Randomization was performed by a statistician who had no involvement in the trial and merely assigned a number to each patient. Capsules of resveratrol and placebo were identical. A person, who did not take part in our study, was asked to prepare the bottles for two groups by putting the tablets of resveratrol and placebo into identical bottles and then apply codes with the number of each patient. Both the researchers and participants were blinded from the time of randomization until analysis was completed.

### 2.5. Outcomes

The primary objective of the study was to assess the differences in changes in the percentage of $CD14^{+}CD16^{+}$ monocytes, circulating levels of TNF-α, IL-6, IL-1β, and MCP-1, cytokine secretion from LPS-treated PBMCs, as well as NF-κB, TLR2, and TLR4 gene expression from baseline to the end of the trial in patients treated with 800 mg/day resveratrol versus patients

treated with placebo. The secondary objective was to assess the differences in changes from baseline to the end of the trial of blood pressures, weight, body mass index (BMI), waist circumference (WC), hip circumference, waist to hip ratio (W/H ratio), fasting blood glucose (FBS), glycated hemoglobin (HbA1c), insulin, homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), total cholesterol, LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triacylglycerol (TG), urea, uric acid, creatinine, total protein, serum glutamate oxaloacetate transaminase (SGOT), and serum glutamate pyruvate transaminase (SGPT) in patients treated with resveratrol versus patients treated with placebo.

### 2.6. Intervention

Patients in the resveratrol group received 400 mg twice a day of resveratrol capsules (99% pure trans-resveratrol; Mega resveratrol, UK) for a period of eight weeks. The placebo group received 400 mg twice a day of placebo capsules (completely inert microcellulose) supplementation for the same period of time. Resveratrol and placebo capsules were identical in size, shape, color, and taste. All patients were allowed to continue their existing hypoglycemic treatment during the course of the study but were instructed to abstain from consuming significant amounts of resveratrol-rich foods and beverages. Each participant was asked to complete a questionnaire during the study to monitor their health status and the use of drugs or supplements. All patients were asked to maintain the same diet during the study. A dietician provided nutritional guidance to all subjects on a weekly basis, analyzed meals, and suggested changes if necessary.

### 2.7. Adverse events and compliance

Adverse events and compliance during the study were monitored by phone calls on the weekly basis. Participants were instructed to inform the researchers if adverse effects or exposure to insulin treatment occurred. To assess the compliance, participants returned unused capsules at the end of the trial and the pill counting was performed.

### 2.8. Physical measurements

Blood pressure was measured using a mercury sphygmomanometer according to standard protocol [19]. Subjects were seated at rest for at least 10 min, and two measurements were taken at five-min intervals. Height and weight were measured using a stadiometer and a standard weighing scale, respectively. Heavy outer garments and shoes were removed before height and weight measurements. BMI was calculated as weight in kilograms divided by the square of height in meters. WC was measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest [20]. Hip circumference was measured at its widest portion of the buttocks at left and at the greater trochanters at right [20].

### 2.9. Biochemical variables and inflammatory markers

Fasting blood samples (12 h) were taken at the baseline and after eight weeks of treatment. All samples were promptly centrifuged and plasma was separated and kept frozen at -80℃until used. Biochemical variables including FBS, total cholesterol, LDL-C, HDL-C, TG, urea, uric acid, creatinine, total protein, SGOT, SGPT, and high-sensitivity C-reactive protein (hsCRP) were measured using enzymatic methods by BIOLIS 24i Premium autoanalyzer (Tokyo Boeki Machinery Ltd., Japan). HbA1c was measured with HPLC method by Tosoh G8 instrument (South San Francisco, CA). Insulin was assessed by Insulin AccuBind enzyme-linked

immunosorbent assay (ELIZA) Kit (Monobind Inc, California, USA). Plasma levels of inflammatory markers (TNF-α, IL-6, IL-1β, and MCP-1) (Abcam, Cambridge, MA) were determined by ELIZA, according to manufacturer's instructions. All samples were tested at least in duplicates.

### 2.10. PBMCs isolation and treatment

Peripheral blood mononuclear cells (PBMCs) isolation was carried out under sterile conditions to avoid monocytes activation. Blood was diluted (1:1) with phosphate buffer solution (PBS) and centrifuged by density gradient with Lympholyte (Cedarlane, Ontario, Canada) according to the manufacturer's instructions. After washing the PBMCs (2×) with PBS, the cells were stored at -80℃prior to RNA extraction [21].

In order to treat the PBMCs, $1\times10^6$ cells were cultivated in RPMI 1640, supplemented with 5% fetal bovine serum (FBS; GIBCO BRL, Rockville, MD, USA), 50 U/ml penicillin, and 50 µg/ml streptomycin. The cells were then treated with 100 ng/mL of LPS (Sigma, Saint Louis, Missouri, USA) for 24 h [22].

### 2.11. Flow cytometry analysis

Heparinized whole blood (100 µL) was incubated for 15 min in the dark at room temperature with 5µL of fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibody (FITC anti-human CD14 antibody; Biolegend, San Diego, CA) and 5µL of phycoerythrin (PE)-labeled anti-CD16 antibody (PE anti-human CD16 antibody; Biolegend, San Diego, CA). Then, another aliquot of the same sample (100 µL) was stained with 5µL of FITC-labeled anti-CD14 mouse IgG1 antibody and 5µL of PE-labeled anti-CD16 mouse IgG1 antibody as an isotype control. After staining, 2 mL of ammonium chloride solution was added to each tube. Next, the samples were

incubated for 15 min in the dark at room temperature, washed twice with PBS, and centrifuged at 1500 rpm for 10 min. The cells were resuspended in PBS for flow cytometric analysis [23]. Data were collected on a Partec PAS and were analyzed using FloMax software. At least, 10,000 events were counted per analysis.

### 2.12. Quantitative real-time PCR

Total RNA from PBMCs was isolated using GeneAll Hybrid-R RNA purification kit (GeneAll Biotechnology Co., Seoul South Korea). RNA was reverse transcribed into cDNA using RevertAid RT Reverse Transcription Kit (Thermofisher, USA).

Changes in the expression of several selected target genes were further validated by quantitative RT-PCR in duplicates using SYBR Green RealQ Plus 2x Master Mix Green (Ampliqon) on Corbett Rotor-Gene 6000 Light Cycler (Qiagen, Hilden, Germany). The data were normalized to the β-actin transcript level. The delta-delta Ct method was used to calculate the relative expression. The sequences of the primers used are shown in the supplementary file.

### 2.13. Statistical analyses

Sample size calculation was based on changes in the mean value of the percentage of $CD14^{+}CD16^{+}$ monocytes as a primary outcome. To detect a 25% change in the primary outcome at a two-sided 0.05 significance level with a power of 0.8, 19 participants were required in each group. Because we anticipated a 25% dropout rate, a final sample size of 24 patients per group were recruited in this study.

All analyses were performed with SPSS 22.0 (SPSS Inc., Chicago, USA). Qualitative data are shown as proportions. Quantitative data are expressed as the mean value ± SD or as the median and the 25th to the 75th interquartile range. For RT-PCR results, the statistical analyses were

applied to the normalized Ct values: to the endogenous control actin (Ct target – Ct β actin). Assumptions of normality and equal variances were checked to perform the appropriate statistical test. Variables with skewed distribution were logarithmically transformed prior to analysis. Baseline data were analyzed to determine the possible significant starting inter-group differences. A paired t-test and independent t-test were used for numerical analysis of the normally distributed data. The Wilcoxon signed-rank test and Mann-Whitney U test were used for nonparametric distributions. To account for differences between the arms of the analyzed variables at enrollment, comparisons of change from baseline between the resveratrol and placebo arms were performed by one-way analysis of covariance (ANCOVA) to adjust for baseline imbalances of the analyzed endpoints. The covariates used for adjustment were age, duration of diabetes, age of onset of diabetes, BMI, hypercholesterolemia, and type of drugs consumption. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

The flow diagram of the trial is shown in Fig. 1. A total of 45 subjects completed the study. At the baseline, 49 participants were randomly assigned to the resveratrol group (n = 25) and placebo group (n = 24). Of the 49 participants, four from the placebo arm were dropped out.

### 3.1.Compliance, tolerability, and safety

Compliance assessment was based on pill counts. The compliance rates were 96% and 93% in the resveratrol and placebo groups, respectively. Resveratrol was well tolerated and no serious adverse effects were observed in participants.

### 3.2.Baseline characteristics

Baseline characteristics of the participants are outlined in Table 1. At the baseline level, there was no significant difference among the variables between two groups except in the cases of total cholesterol and HOMA-IR. Total cholesterol and HOMA-IR levels were significantly higher in the resveratrol group compared to the placebo group.

### 3.3.Effects of resveratrol on inflammatory parameters

Differences between two groups in assessed inflammatory parameters at the end of eight weeks of the trial are shown in Table 2. We first investigated circulating $CD14^{+}CD16^{+}$ monocyte subsets using flow cytometry. This study revealed that the percentage of $CD14^{+}CD16^{+}$ monocytes did not significantly differ between resveratrol and placebo groups. Neither the change in plasma levels of cytokines (TNF-α, IL-6, and IL-1β) nor the change in LPS-stimulated levels of TNF-α, IL-6, and IL-1β from PBMCs differed from zero to eight weeks between resveratrol and placebo groups. Although a significant decrease in plasma level of MCP-1 was observed in the resveratrol and placebo groups compared to their baselines, the change in the mean difference of MCP-1 was not significant when the two groups were compared. We also found that there was not a significant difference in the change of WBC count in two study groups. We further analyzed the changes in the transcript levels of NF-κB, TLR2, and TLR4. We found that the transcript levels of these genes did not differ between resveratrol and placebo groups. The mean hsCRP level was lower in the resveratrol group compared with the control group (3.63 ±1.99 vs 4.08± 3.18 mg/L) from pre- to post-intervention, but it was not statistically significant.

### 3.4.Effects of resveratrol on metabolic and anthropometric parameters

Metabolic and anthropometric parameters of the subjects at the baseline and after eight weeks of resveratrol supplementation or placebo are shown in Table 3. A significantly greater decrease in the mean plasma glucose level was found in the resveratrol group (-31.84 ± 47.6 mg/dl) compared with the placebo group (-2.95 ± 31.78 mg/dl, P = 0.048). There were no significant differences in HbA1c levels between the two groups. Changes in serum insulin, HOMA-IR, and HOMA-β levels were not significantly different between the two groups. We also observed no significant differences in TG, total cholesterol, SGOT, SGPT, urea, uric acid, creatinine, and total protein levels between the two groups. Although a significant decrease in LDL-C level was observed in the resveratrol and placebo groups compared to their baselines, there was no significant mean difference of LDL-C between the resveratrol and placebo groups. In the resveratrol group, the reduction of HDL-C level was observed, however, no difference was found when the mean difference of HDL-C level was compared between the resveratrol and placebo groups.

We also analyzed the effect of resveratrol supplementation on anthropometric parameters. WC was not significantly modified after resveratrol supplementation. Significant decreases in weight, BMI, hip circumference, and W/H ratio were observed in the resveratrol group compared to their baseline; however, the changes in the mean differences of these parameters were not significant between the resveratrol and placebo groups. A significant reduction in systolic (-10.42 ± 8.40 mmHg vs. -1.475 ± 8.72 mmHg, P=0.002) and diastolic (-5.6 ± 6.50 mmHg vs. 1.50 ± 8.75 mmHg, P=0.006) blood pressures were observed in resveratrol group compared to placebo (Table 3). Since 50% of the patients were on anti-hypertensive medication, we further analyzed the effect of resveratrol supplementation on blood pressure in treated and untreated groups,

separately. Sub-group analysis showed that the beneficial effect of resveratrol on blood pressure is uniform across both sub-groups (P = 0.000 for SBP and 0.003 for DBP) (Data not shown).

## 4. Discussion

Based on the data from in vitro and in vivo studies, resveratrol exerts the anti-inflammatory effects via modulation of key players involved in the inflammatory responses [24, 25]. However, clinical trials addressing the anti-inflammatory effects of resveratrol are still limited and challenging. A number of clinical trials have demonstrated the anti-inflammatory effect of resveratrol in different pathological conditions [17, 18, 26, 27]. It appears that the topic is highly complex as some studies failed to show any anti-inflammatory effect of resveratrol [28-32]. In this context, the present study adds to the knowledge about the anti-inflammatory effects of resveratrol supplementation in patients with T2D.

In the present study, we collected the data from a set of inflammatory markers. As a first step, we measured changes in the relative percentages of monocyte subset ($CD14^{+}CD16^{+}$) that has been implicated in the pathogenesis of several infectious and metabolic diseases including chronic liver disease, atherosclerosis, and T2D [33]. In addition, we evaluated LPS-stimulated cytokine release from PBMCs as a convenient and simple surrogate approach to characterize changes in immune function [34]. To investigate more specifically the relationship between resveratrol consumption and inflammation, we assessed the expression level of key genes involved in inflammation. We also measured hsCRP as a nonspecific inflammatory marker, elevated levels of which have been strongly associated with chronic systemic inflammation. This array of inflammatory markers has allowed us to better understand the possible effect of

resveratrol on inflammation in patients with T2D. The data of this study demonstrated that resveratrol consumption in patients with T2D for eight weeks had no beneficial effects on inflammatory markers. More specifically, our double-blinded randomized study demonstrated that a) the percentage of $CD14^{+}CD16^{+}$ monocytes, b) the levels of inflammatory cytokines and chemokines including TNF-α, IL-6, IL-1β, hsCRP, and MCP-1, c) cytokine secretion capacity of PBMCs under LPS-stimulated condition, d) the expression of genes involved in the inflammatory response were not significantly altered in T2D patients following resveratrol consumption. These findings suggest that resveratrol supplementation is not capable of reducing pro-inflammatory markers in patients with T2D. In support of these findings, Bo et al. [35] showed that six months resveratrol supplementation has no effect on serum inflammatory markers (hs-CRP and IL-6) in patients with T2D. Zare Javid et al. [36] displayed that resveratrol supplementation (480 mg/day) for four weeks does not alter serum inflammatory cytokines (IL-6 and TNF-α) in T2D patients with periodontal disease. In another clinical study, Crandall et al. [37] reported that four weeks of resveratrol supplementation has no effect on the hsCRP marker in impaired glucose tolerance individuals. More importantly, a meta-analysis of data from 10 randomized clinical trials did not support a significant effect of resveratrol supplementation on the plasma concentration of CRP [38]. In contrast to these findings, Carneiro et al. showed the anti-inflammatory properties following resveratrol consumption [39]. They showed that one-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in PBMCs of T2D patients [39]. The expression of the pro-inflammatory cytokines IL-1β and TNF-α was significantly reduced in PBMCs of patients taking grape extract containing resveratrol. They did not find either any significant change in serum inflammatory markers except for a significant reduction of IL-6 level [39]. It is of note

that Carneiro et al. did not use a pure resveratrol and, therefore, a synergistic action between resveratrol and other polyphenolic compounds in the grape extract can be questioned. In addition, resveratrol has been shown to have anti-inflammatory effects in patients with non-alcoholic fatty liver disease and healthy obese men [18, 28, 40]. Poulsen et al. found no significant effect of resveratrol on the expression of inflammatory genes in subcutaneous adipose tissue [41]. Taken together, the data from our comprehensive study and the others provide the evidence that the findings are not entirely consistent and the anti-inflammatory property of resveratrol needs to be cautiously used in patients with T2D. Although the mechanism for the lack of an anti-inflammatory property of resveratrol in this study is unknown, it could be attributed to the short-time period of resveratrol supplementation in the present study. Given that obesity triggers a chronic low-grade inflammation status, it seems that a long-term supplementation of resveratrol is required to observe significant changes in obesity-associated inflammation in patients with T2D. Furthermore, we suggest that the effect of resveratrol on inflammation is performed in patients with a lower diabetes duration. Because subclinical chronic inflammation is a common feature in the natural history of diabetes, long-lasting diabetes, as observed in the current study with an average of nine years of the disease, with its inveterate chronic low-grade pro-inflammatory status does not seem to be sensitive to this supplementation.

Our study demonstrated that resveratrol supplementation in patients with T2D for eight weeks led to a significant decrease in FBS level. Several mechanisms including increased SIRT1 activity and increased the expression of GLUT4 have been proposed to explain the beneficial effect of resveratrol on glucose control [42, 43]. Resveratrol supplementation had no significant effect on any of the other parameters assessed. Several studies included measurements of

biochemical parameters after resveratrol supplementation [44-46]. Hausenblas et al. in a meta-analysis of data from six unique datasets examining a total of 196 T2D patients (104 resveratrol, 92 control/placebo) demonstrated statistically significant effects of resveratrol supplementation on systolic blood pressure, HbA1c, and creatinine, but not for fasting glucose, HOMA-IR, diastolic blood pressure, insulin, TG, LDL-C, and HDL-C [47]. Sahebkar et al. in a meta-analysis revealed that resveratrol supplementation did not alter plasma levels of total cholesterol, LDL-C, HDL-C, TG, and glucose [48]. A recent meta-analysis also demonstrated that resveratrol supplementation did not change blood glucose, insulin, TG, and LDL levels in patients with non-alcoholic fatty liver disease [49]. Our data revealed that weight and BMI tended to decrease upon resveratrol treatment. In vitro and in vivo studies have revealed that this effect of resveratrol is mediated through the suppressing the feeding center [50], inhibiting adipogenesis [50], increasing fatty acid oxidation [51], and inducing the formation of the brown-like adipocyte in white adipose tissue [52]. Given the close link between the obesity and chronic inflammation, our findings of reduced body weight and no changes of the inflammatory markers upon resveratrol supplementation are somehow unexpected. No significant effect of resveratrol consumption on the level of inflammatory markers (despite a reduction in body weight in resveratrol group) might be attributed to the effect of resveratrol on abdominal fat. It is believed that BMI does not recognize fat distribution and the accumulation of fat in and around the abdominal region associated with obesity. In fact, recent research suggests that measures of WC correlate with visceral adiposity and it is generally more accurate than BMI and weight in explaining obesity-related health risk in T2D patients [53]. No significant effect of resveratrol on WC level in the present study supports our finding that resveratrol has no anti-inflammatory effect in patients with T2D. In addition, the current study suggests that consumption of

resveratrol had a favorable effect on systolic and diastolic blood pressures. In line with our result, a recent clinical trial investigating the effect of resveratrol on hypertension showed that resveratrol is able to reduce blood pressure to normal levels [54]. Moreover, consistent with our results, a recent meta-analysis of six randomized controlled trials investigating the effect of resveratrol on blood pressure reported that resveratrol significantly reduces blood pressure [55]. The improvement in blood pressure following resveratrol consumption may result from its ability to activate adenosine monophosphate-activated protein kinase (AMPK), which directly phosphorylates eNOS leading to increasing NO production [56]. Furthermore, resveratrol has been shown to act as an endothelin (ET)-1 antagonist and downregulate the concentration of angiotensin (Ang) II; two substances that have been found to play roles in the development of hypertension [56].

Our study had some strengths and limitations. We collected a homogeneous sample of well-characterized diabetic patients, which increases sensitivity for detecting the associations. Our study also benefits from 800 mg/day resveratrol supplementation during eight weeks compared to the dosages of resveratrol supplementation (40 mg, 250 mg, and 500 mg) in the other studies [35, 57]. The selected dose of resveratrol was almost similar to resveratrol supplementation doses in Movahed et al. [45] and Thazhath et al. [58] studies. Also, it was well tolerated without showing any toxic effects in patients with T2D. The limitation of our study included a relatively short-term follow-up period. The study was powered to detect an absolute difference of about 25% between the treatment groups in the percentage of $CD14^{+}CD16^{+}$ as a primary outcome; the standard deviation of $CD14^{+}CD16^{+}$ values in this study was close to 3.97%, whereas our trial was powered to detect mean differences of 1.27% between arms. Thus, the sample size may have been small to unmask a smaller effect in the percentage of $CD14^{+}CD16^{+}$ monocytes; however,

the number of subjects studied was calculated to have 80% power to show a change in the percentage of $CD14^+CD16^+$ if any substantial effect existed. There was no suggestion of any effect on either $CD14^+CD16^+$ monocytes or other inflammatory markers, and thus, it is unlikely that increasing the sample size would have altered our results. Since we did not measure plasma concentration of resveratrol or its metabolites, the actual exposure to resveratrol could not be determined. However, based on phone calls and capsule counts, we believed that a good compliance was achieved in this study.

In conclusion, according to the data presented here, we could not accept our hypothesis that resveratrol supplementation can decrease the inflammatory markers in patients with T2D. Our study demonstrated that eight weeks supplementation with 800 mg/day resveratrol neither has a beneficial immunomodulatory effect nor improves the metabolic patterns of T2D patients except in the case of plasma glucose and blood pressure. More studies with longer durations and different dosages of supplementation are needed to confirm these results.

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**Figure Caption;**

Fig. 1: Flowchart showing recruitment of participants, randomization and completion

**Table 1.** Baseline characteristics of placebo and resveratrol groups

| Variable | Placebo (n = 20) | Resveratrol (n = 25) | P |
|---|---|---|---|
| Age (years) | 61.10 ± 5.61 | 56.48 ± 6.72 | 0.018 |
| Sex (female/male) | 10/10 | 12/13 | 0.894 |
| Duration of diabetes (years) | 11.25 ± 7.41 | 8.64 ± 6.22 | 0.234 |
| Age of onset (years) | 49.85 ± 8.22 | 47.84 ± 9.012 | 0.444 |
| SBP (mmHg) | 128.05 ± 13.30 | 126.30 ± 10.80 | 0.630 |
| DBP (mmHg) | 76.05 ± 8.79 | 77.48 ± 8.72 | 0.627 |
| Weight (kg) | 72.50 (64.77 , 87.72) | 78.50 (72.15 , 94.50) | 0.107 |
| BMI ($kg/m^2$) | 29.13 ± 5.12 | 29.98 ± 3.64 | 0.408 |
| WC (cm) | 101.11 ± 8.82 | 105.12 ± 9.56 | 0.157 |
| Hip circumference (cm) | 105 (102 , 113.50) | 109 (104 , 116) | 0.200 |
| W/H ratio | 0.94 ± 0.05 | 0.94 ± 0.06 | 0.751 |
| FBS (mg/dl) | 138 (126.25 , 166.50) | 160 (124 , 216.50) | 0.189 |
| HbA1c (%) | 7.55 (7.12 , 7.97) | 7.90 (7.3 , 8.85) | 0.156 |
| Urea (mg/dl) | 30.84 ± 7.82 | 33.88 ± 10.74 | 0.758 |
| Creatinine (mg/dl) | 0.99 ± 0.17 | 1 ± 0.19 | 0.801 |
| Uric acid (mg/dl) | 5.44 ± 1.41 | 5.35 ± 1.34 | 0.830 |
| TG (mg/dl) | 132 ± 42.03 | 154.68 ± 66.62 | 0.211 |
| Total cholesterol (mg/dl) | 144.57 ± 28.65 | 163.88 ± 35.29 | 0.048 |
| HDL-C (mg/dl) | 42.65 ± 9.28 | 46.96 ± 8.63 | 0.115 |
| LDL-C (mg/dl) | 78.05 ± 18.42 | 90.16 ± 25.12 | 0.08 |
| Total protein (g/dl) | 6.75 ± 0.60 | 6.90 ± 0.46 | 0.340 |
| SGOT (U/L) | 21 (17 , 29) | 18 (16 , 25.50) | 0.313 |
| SGPT (U/L) | 27.50 (19.50 , 38) | 24 (19 , 31.50) | 0.314 |
| hsCRP (mg/L) | 4.81 ± 2.55 | 4.38 ± 1.73 | 0.471 |
| Insulin (µU/ml) | 13.89 ± 11.41 | 16.44 ± 10.83 | 0.106 |
| HOMA-IR | 5.04 ± 4.58 | 7.21 ± 5.68 | 0.047 |
| HOMA-β | 66.39 ± 47.89 | 65.47 ± 48.04 | 0.553 |
| WBC (1000/µl) | 6.26 ± 1.52 | 6.59 ± 1.40 | 0.425 |
| Neutrophils (%) | 58.15 ± 8.14 | 55.56 ± 8.47 | 0.306 |
| Lymphocytes (%) | 38.45 ± 8.40 | 40.72 ± 8.56 | 0.378 |
| $CD14^+CD16^+$ (%) | 4.96 ± 3.07 | 5.26 ± 4.56 | 0.899 |
| NF-kB | 6.24 ± 1.87 | 6.86 ± 1.85 | 0.471 |
| TLR2 | 8.68 ± 2.24 | 9.46 ± 1.82 | 0.409 |
| TLR4 | 9.68 ± 1.67 | 10.71 ± 1.94 | 0.220 |
| TNF-α(plasma) (pg/ml) | 15.10 ± 5.78 | 12.41 ± 6.63 | 0.247 |
| TNF-α(supernatant) (pg/ml) | 13654 ± 7486 | 11238 ± 7281 | 0.452 |
| IL-1β(plasma) (pg/ml) | 6.42 ± 4.65 | 5.64 ± 3.64 | 0.612 |
| IL-1β(supernatant) (pg/ml) | 4978 ± 3756 | 4010 ± 2767 | 0.515 |
| IL-6(plasma) (pg/ml) | 9.50 ± 7.92 | 7.66 ± 5.42 | 0.653 |
| IL-6(supernatant) (pg/ml) | 165118 ± 65948 | 126133 ± 62023 | 0.181 |
| MCP-1 (pg/ml) | 48.92 ± 14 | 54.58 ± 13.56 | 0.336 |
| Treatment with statins (%) | 80 | 72 | 0.535 |
| Anti-hypertensive drugs (%) | 50 | 56 | 0.686 |
| Metformin use (%) | 100 | 96 | 0.366 |
| Sulphonylureas use (%) | 85 | 76 | 0.453 |

Values are means ± SD for data with normal distribution and median (interquartile ranges) for data not normally distributed. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; W/H ratio, waist to hip ratio; FBS, fasting blood glucose; HbA1c, glycated hemoglobin; TG, triacylglycerol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase; hsCRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; $CD14^{+}CD16^{+}$, cluster of differentiation 14,16; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

**Table 2**. Comparison of changes in the inflammatory parameters during the study period between the placebo and resveratrol groups

| | Placebo (n = 20) | | | | Resveratrol (n = 25) | | | | Resveratrol vs Placebo | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Baseline** | **After 2-months** | **P [a]** | **Absolute change** | **Baseline** | **After 2-months** | **P [a]** | **Absolute change** | **Adjusted mean difference on change from baseline (95%CI)** | **P [b]** |
| WBCs (1000/µl) | 6.26 ± 1.52 | 6.46 ± 1.53 | 0.238 | 0.20 ± 0.80 | 6.59 ± 1.40 | 6.83 ± 1.44 | 0.170 | 0.24 ± 0.84 | 0.03 (-0.55 0.60) | 0.923 |
| $CD14^{+}CD16^{+}$ (%) | 4.96 ± 3.07 | 3.80 ± 2.20 | 0.115 | -1.16 ± 2.59 | 5.26 ± 4.56 | 4.09 ± 2.47 | 0.525 | -1.17 ± 5.54 | -0.17 (-3.28 2.93) | 0.910 |
| NF-kB | 6.24 ± 1.87 | 5.32 ± 1.71 | 0.072 | -0.92 ± 1.42 | 6.86 ± 1.85 | 7.04 ± 1.51 | 0.739 | 0.18 ± 1.67 | 0.83 (-1.22 2.88) | 0.396 |
| TLR2 | 8.68 ± 2.24 | 8.70 ± 2.22 | 0.971 | 0.02 ± 1.28 | 9.46 ± 1.82 | 10.03 ± 1.78 | 0.473 | 0.56 ± 2.40 | 0.51 (-1.70 2.73) | 0.623 |
| TLR4 | 9.68 ± 1.67 | 9.78 ± 1.54 | 0.768 | 0.1 ± 1.09 | 10.71 ± 1.94 | 10.53 ± 1.19 | 0.783 | -0.17 ± 1.95 | -0.45 (-2.57 1.68) | 0.655 |
| TNF-α (plasma) (pg/ml) | 15.10 ± 5.78 | 14.71 ± 5.48 | 0.390 | -0.39 ± 1.70 | 12.41 ± 6.63 | 12.28 ± 6.30 | 0.812 | -0.13 ± 2.08 | 0.65 (-1.07 2.37) | 0.439 |
| TNF-α (supernatant) (pg/ml) | 13654 ± 7486 | 13732 ± 11421 | 0.980 | 78.39 ± 9927 | 11238 ± 7281 | 13552 ± 10639 | 0.344 | 2313 ± 7728 | -1238.7 (-8278.54 5801.16) | 0.712 |
| IL-1β (plasma) (pg/ml) | 6.42 ± 4.65 | 6.25 ± 4.04 | 0.712 | -0.17 ± 1.78 | 5.64 ± 3.64 | 5.73 ± 3.04 | 0.802 | 0.09 ± 1.42 | 0.15 (-1.32 1.62) | 0.836 |
| IL-1β (supernatant) (pg/ml) | 4978 ± 3756 | 4796 ± 4260 | 0.826 | -181.76 ± 2404.18 | 4010 ± 2767 | 4995 ± 2573 | 0.189 | 985 ± 2320 | 1164.62 (-1378.62 3707.85) | 0.338 |
| IL-6 (plasma) (pg/ml) | 9.50 ± 7.92 | 6.21 ± 5.23 | 0.223 | -3.29 ± 7.96 | 7.66 ± 5.42 | 7.05 ± 3.98 | 0.632 | -0.61 ± 4.31 | 0.012 (-5.92 5.95) | 0.996 |
| IL-6 (supernatant) (pg/ml) | 165118 ± 65948 | 182127 ± 89021 | 0.364 | 17009 ± 52967 | 126133 ± 62023 | 138822 ± 44071 | 0.300 | 12688 ± 40406 | -19068.55 (-55278.06 17140.96) | 0.276 |
| MCP-1 (pg/ml) | 48.92 ± 14 | 43.78 ± 13.78 | 0.048 | -5.13 ± 7.53 | 54.58 ± 13.56 | 47.09 ± 11.93 | 0.009 | -7.49 ± 8.21 | -1.665 (-11.4 8.06) | 0.720 |
| hsCRP (mg/L) | 4.81 ± 2.55 | 4.08 ± 3.18 | 0.795 | -0.73 ± 3.16 | 4.38 ± 1.73 | 3.63 ± 1.99 | 0.058 | -0.74 ± 1.86 | -0.15 (-1.86 1.56) | 0.860 |

Values are means ± SD. WBCs, white blood cells; $CD14^{+}CD16^{+}$, cluster of differentiation 14,16; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha; IL, interleukin; MCP-1, monocyte chemoattractant protein-1. hs-CRP, high-sensitivity C-reactive protein.

P [a] values indicate comparison within groups.

P [b] values indicate comparison between the changes of each variable between two groups.

**Table 3**: Comparison of changes in the anthropometric, and biochemical parameters during the study period between the placebo and resveratrol groups.

| | Placebo (n = 20) | | | | Resveratrol (n = 25) | | | | Resveratrol vs Placebo | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Baseline** | **After 2-months** | **P [a]** | **Absolute change** | **Baseline** | **After 2-months** | **P [a]** | **Absolute change** | **Adjusted mean difference on change from baseline (95%CI)** | **P [b]** |
| SBP (mmHg) | 127.50 (120 , 140) | 130 (120.05 , 130.40) | 0.529 | -1.475 ± 8.72 | 129 (120 , 131.75) | 120 (110 , 120.75) | <0.001 | -10.42 ± 8.40 | -9.350 (-15 -3.70) | 0.002 |
| DBP (mmHg) | 73.50 (70 , 80.47) | 80 (70.20 , 80.92) | 0.439 | 1.50 ± 8.75 | 80 (70.05 , 80.40) | 70 (70 , 76.50) | 0.001 | -5.6 ± 6.50 | -7.362 (-12.43 -2.29) | 0.006 |
| Weight (kg) | 72.50 (64.77 , 87.72) | 72.85 (65.25 , 88.75) | 0.545 | 0.19 ± 2.02 | 78.50 (72.15 , 94.50) | 77.30 (69.50 , 92.50) | 0.006 | -1.70 ± 2.74 | -1.41 (-3.0 0.19) | 0.082 |
| BMI (kg/m$^2$) | 29.13 ± 5.12 | 29.21 ± 5.11 | 0.667 | 0.075 ± 0.75 | 29.98 ± 3.64 | 29.36 ± 3.75 | 0.007 | -0.62 ± 1.01 | -0.52 (-1.10 0.07) | 0.083 |
| WC (cm) | 101.11 ± 8.82 | 100.95 ± 9.73 | 0.733 | -0.15 ± 2.94 | 105.12 ± 9.56 | 104.36 ± 9.11 | 0.142 | -0.76 ± 2.50 | -0.47 (-2.357 1.42) | 0.617 |
| Hip circumference (cm) | 105 (102 , 113.50) | 104.50 (100.50 , 112.50) | 0.06 | -1.35 ± 2.79 | 109 (104 , 116) | 107 (103 , 114) | 0.001 | -2.04 ± 2.55 | -0.44 (-2.16 1.29) | 0.612 |
| W/H ratio | 0.94 ± 0.05 | 0.95 ± 0.06 | 0.170 | 0.01 ± 0.03 | 0.94 ± 0.06 | 0.96 ± 0.056 | 0.035 | 0.01 ± 0.03 | -0.001 (-0.02 0.02) | 0.921 |
| FBS (mg/dl) | 138 (126.250 , 166.50) | 136 (120 , 158) | 0.654 | -2.95 ± 31.78 | 160 (124 , 216.50) | 130 (107 , 175.50) | 0.005 | -31.84 ± 47.6 | -27.24 (-54.16 -0.31) | 0.048 |
| HbA1c (%) | 7.55 (7.12 , 7.97) | 7.35 (7.02 , 8.05) | 0.247 | -0.135 ± 0.51 | 7.90 (7.30 , 8.85) | 8 (6.75 , 8.95) | 0.136 | -0.248 ± 0.84 | -0.08 (-0.57 0.41) | 0.745 |
| Urea (mg/dl) | 30.84 ± 7.82 | 31.47 ± 8.61 | 0.620 | 0.25 ± 5.52 | 33.88 ± 10.76 | 31.96 ± 11.33 | 0.173 | -1.92 ± 9.08 | -3.05 (-7.72 1.63) | 0.195 |
| Creatinine (mg/dl) | 0.99 ± 0.17 | 0.96 ± 0.19 | 0.088 | -0.02 ± 0.07 | 1 ± 0.19 | 1 ± 0.20 | 0.413 | -0.01 ± 0.09 | 0.02 (-0.03 0.06) | 0.534 |
| Uric acid (mg/dl) | 5.44 ± 1.41 | 5.43 ± 1.31 | 0.910 | -0.01 ± 0.46 | 5.35 ± 1.34 | 5.26 ± 1.37 | 0.579 | -0.09 ± 0.78 | -0.2 (-0.62 0.22) | 0.345 |
| TG (mg/dl) | 132 ± 42.03 | 112.55 ± 35.65 | 0.111 | -19.44 ± 49.02 | 154.68 ± 66.62 | 152.76 ± 66.88 | 0.994 | -1.92 ± 67.19 | 27.39 (-8.02 13.33) | 0.127 |
| Total cholesterol (mg/dl) | 144.57 ± 28.65 | 137.42 ± 17.42 | 0.312 | -7.15 ± 27.15 | 163.88 ± 35.29 | 155.52 ± 26.78 | 0.180 | -8.36 ± 30.24 | 10.94 (-2.95 24.83) | 0.119 |
| HDL-C (mg/dl) | 42.65 ± 9.28 | 41.55 ± 9.95 | 0.325 | -1.10 ± 4.86 | 46.96 ± 8.63 | 45 ± 8.48 | <0.001 | -1.96 ± 2.28 | -0.19 (-2.55 2.16) | 0.870 |
| LDL-C (mg/dl) | 78.05 ± 18.42 | 69.20 ± 11.56 | 0.029 | -8.85 ± 16.77 | 90.16 ± 25.12 | 80.28 ± 17.10 | 0.032 | -9.88 ± 21.54 | 2.65 (-13.08 12.55) | 0.617 |
| Total protein (g/dl) | 6.75 ± 0.6 | 6.79 ± 0.56 | 0.780 | 0.040 ± 0.63 | 6.90 ± 0.46 | 7.08 ± 0.32 | 0.096 | 0.176 ± 0.50 | 0.12 (-0.25 0.50) | 0.515 |
| SGOT (U/L) | 21 (17 , 29) | 19 (17 , 22) | 0.082 | -2.52 ± 7.26 | 18 (16 , 25.50) | 20 (17 , 24.50) | 0.866 | -1.08 ± 8.87 | 1.41 (-4.25 7.06) | 0.617 |
| SGPT (U/L) | 27.50 (19.50 , 38) | 24 (20 , 28.25) | 0.052 | -5.95 ± 12 | 24 (19 , 31.50) | 23 (18.50 , 30) | 0.710 | -0.8 ± 9.10 | 4.78 (-2.11 11.67) | 0.168 |
| Insulin (μU/mL) | 13.89 ± 11.41 | 12.05 ± 11.26 | 0.949 | -1.84 ± 6.01 | 16.44 ± 10.83 | 13.15 ± 8.92 | 0.006 | -3.29 ± 6.16 | -1.88 (-5.31 1.55) | 0.274 |
| HOMA-IR | 5.04 ± 4.58 | 4.68 ± 4.45 | 0.620 | -0.36 ± 1.03 | 7.21 ± 5.68 | 5.57 ± 3.57 | 0.003 | -1.64 ± 2.58 | -1.08 (-2.36 0.19) | 0.094 |
| HOMA-β | 66.39 ± 47.89 | 63.28 ± 60.38 | 0.606 | -3.11 ± 20.85 | 65.47 ± 48.04 | 77.97 ± 75.13 | 0.412 | 12.50 ± 53.03 | 5.97 (-21.08 33.01) | 0.658 |

Values are means ± SD for data with normal distribution and median (interquartile ranges) for data not normally distributed. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; W/H ratio, waist to hip ratio; FBS, fasting blood glucose; HbA1c, glycated hemoglobin; TG, triacylglycerol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; SGOT, serum glutamate oxaloacetate transaminase; SGPT,

serum glutamate pyruvate transaminase; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function.

P [a] values indicate comparison within groups.

P [b] values indicate comparison between the changes of each variable between two groups.

Fig. 1: