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Long-term treatment with resveratrol attenuates oxidative stress pro-inflammatory mediators and apoptosis in streptozotocin-nicotinamide-induced diabetic rats

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Abstract. This study was designed to investigate the possible effectiveness of chronic resveratrol administration on redox state, inflammatory mediators and apoptosis rate in diabetic rats. Male Wistar rats were divided into four groups ($n = 6$): normal control, diabetic control, normal rats treated with resveratrol, and diabetic rats treated with resveratrol. Diabetes was induced by injection of streptozotocin (50 mg/kg; *i.p.*), 15 min after the prescription of nicotinamide (110 mg/kg; *i.p.*) in 12 h-fasted rats. Four-month oral resveratrol administration (5 mg/kg/day) significantly alleviated hyperglycemia, weight loss, enhancement of oxidative markers (lipid peroxidation index, nitrite/nitrate content and oxidized to reduced glutathione ratio) and superoxide dismutase activity in diabetic rats. Moreover, resveratrol administration to diabetic rats improved the elevated levels of plasma TNFα and IL-6 as well as NF-κB activity of polymorphonuclear cells. On the other hand, four months resveratrol administration decreased the apoptosis rate in the kidney, heart, retina, sciatic nerve and the polymorphonuclear cells of diabetic rats. These beneficial antidiabetic observations suggest that treatment with resveratrol may be considered as a therapeutic approach to reduce diabetic-related complications.

Key words: Diabetes — Antioxidant — Hyperglycemia — Redox state — Cell death

Abbreviations: DC, diabetic control rats; DTR, diabetic rats treated with resveratrol; GSH, glutathione; GSSG, oxidized glutathione; HbA1c, glycosylated hemoglobin; IL-6, interleukin 6; NAD, nicotinamide adenine denucleotide; NC, normal control rats; NF-κB, nuclear factor kappa B; NIDDM, non insulin dependent diabetic mellitus; NTR, normal control rats treated with resveratrol; OD, optic density; $O_2^{\bullet -}$, superoxide radical; PMNs, polymorphonuclear cells; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substrate; TNFα, tumor necrosis factor alpha.

Introduction

Diabetes mellitus, a chronic and progressive metabolic disorder, is a challenging public health problem and nowadays, diabetes-related complications are one of the most important contributing mortality factors in the world (Dailey 2011). Diabetes-related hyperglycemia which results from insufficient secretion or action of endogenous insulin, can induce oxidative stress and its related inflammation *via* enhancements in glucose oxidation, advanced glycation end products, protein kinase C, hexosamine and polyol pathways fluxes and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6) and nuclear factor kappa B (NF-κB) activity (Rains and Jain 2011). It widely has been accepted that oxidative stress, an imbalance

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between production and detoxification of oxygen/nitrogen-free radicals, plays a key role in the onset and development of diabetes complications. Peroxidation or glycation of lipids, proteins, and DNA, reduction of antioxidants defenses and progression of tissues inflammations are some disturbances, which are induced by oxidative stress (Rains and Jain 2011). Many of the above hyperglycemia-induced pathways converge to activate NF-κB, which in turn contributes to increase pro-inflammatory cytokines productions, oxidative stress worsening and programmed cell death (apoptosis) propelling (Singh et al. 2011).

During the past decades, some approaches (such as diet, exercise, insulin therapy, and antidiabetic drugs) have provided to diminish diabetes complications. In order to antidiabetic drugs side effects such as hypoglycemia, diarrhea, hepatotoxicity, dyslipidemia, lactic acidosis and hypercoagulability (Palsamy and Subramanian 2008), there is a great need to focus on additional therapeutics with negligible adverse effects, which would improve diabetic patients health problems.

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene), a polyphenolic phytoalexin found in different plants such as grapes, peanuts and berries, has several beneficial properties such as lifespan extending, antioxidant, anti-inflammatory, anticancer, anticoagulant, cardioprotective and vasoprotective effects (Szkudelska and Szkudelski 2010; Csiszar 2011; Lee et al. 2011). In regard to the central role of oxidative stress and proinflammatory cytokines in the pathogenesis of diabetes, in the recent years, numerous investigations have focused on the role of resveratrol in prevention or treatment of diabetes complications (Szkudelska and Szkudelski 2010; Csiszar 2011; Sharma et al. 2011). In this regard, it has been reported that short-term treatment with resveratrol (2–8 weeks) has beneficial antidiabetic effects, mainly *via* reduction in blood glucose, lipid peroxidation, circulatory proinflammatory cytokines and apoptosis levels with concomitant enhancement of antioxidants defenses (Kumar 2007; Sharma et al. 2009, 2011; Palsamy and Subramanian 2010; Zhang et al. 2010; Lee et al. 2011). On the other hand, to date, no serious side effects were reported for long-term resveratrol treatment in healthy subjects during *in vitro* and *in vivo* studies (Cottart et al. 2010).

Currently, resveratrol has become available in pill forms as a dietary supplement and based on previous studies, it seems that short-term prescription of resveratrol to be useful, safe, and well tolerable. In order to poor information about long-term administration of resveratrol in chronic disorders such as diabetes mellitus and cancers, a requirement to further investigations to determine its efficacy for the treatment of diabetic patients, has been previously suggested (Cottart et al. 2010). The present study was designed to evaluate whether chronic resveratrol administration (4 months) can also attenuate diabetes complications in streptozotocin (STZ)-nicotinamide model of diabetic rat. In this study, we evaluated the plasma levels of TNFα and IL-6 as well as polymorphonuclear cells (PMNs) NF-κB activity for determination of resveratrol anti-inflammatory effect. For assessing redox state, we measured the levels of lipid peroxidation, nitrite/nitrate, oxidized to reduced glutathione ratio (GSSG/GSH), and superoxide dismutase (SOD) activity in the blood samples. We also determined apoptosis rate in PMNs and some tissues usually affected by diabetes mellitus including kidney, liver, heart, aorta, and sciatic nerve.

Materials and Methods

Experimental design

Male Wistar rats (Razi Institute, Tehran, Iran) weighing 320–350 g were housed at room temperature (22–25°C) with 12:12-h light/dark cycles and free access to food and water. Rats were randomly divided into four groups (6 in each): normal control (NC), diabetic control (DC), normal control treated with resveratrol (NTR), and diabetic treated with resveratrol (DTR). The study protocol was designed in accordance with NIH guidelines for the care and use of laboratory animals and based on the method previously described by Palsamy and Subramanian (2008). Diabetes was induced by injection of STZ (50 mg/kg *i.p.*) dissolved in 0.1 M of citrate buffer (pH 4.5), 15 min after the administration of nicotinamide (110 mg/kg; *i.p.*) in 12 h-fasted rats (Figure 1). Citrate buffer was injected alone in control rats. Nicotinamide preserves the pancreatic β-cells (up to 40%) from STZ cytotoxicity and produces non-insulin dependent diabetes (NIDDM) similar to human NIDDM (Masiello et al. 1998). To prevent from the fatal hypoglycemic effect of pancreatic insulin release, 10% glucose solution was provided for the rats 6 h after STZ injection for the next 24 h. After 48 h blood glucose levels were measured using glucometer (Arkray, Kyoto, Japan) and the rats with blood glucose levels higher than 14 mM were included to the protocol as diabetic rats. Body weight and blood glucose level were measured

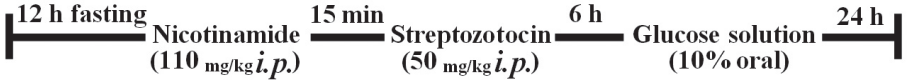

Figure 1. The scheme of experimental procedures.

monthly for four months. Resveratrol treatment (5 mg/kg; minimum dose with anti-hyperglycemic and non-toxic effects) was carried out orally in aqueous solution for four months (Palsamy and Subramanian 2008). The dosage was regulated every week. At the end of experimental period, fasted rats were anesthetized with ketamine (80 mg/kg) and killed by cervical decapitation. Blood samples (5 ml/rat) were collected from retro-orbital sinus and then intended tissues (left kidney, liver, left ventricle, descending aorta and the left sciatic nerve) quickly were removed, rinsed in cold saline and freezed at −80°C until measurements. All manipulations were held in the morning.

All above chemicals (except resveratrol) were purchased from Sigma (St. Louis, MO, USA). Resveratrol was obtained from Cayman chemicals (Cayman chem., Ann Arbor, MI, USA).

Estimation of redox status

Lipid peroxidation was measured spectrophotometrically at 535 nm using thiobarbituric acid-reacting substance (TBARS) production as previously described (Draper and Hadly 1990). SOD activity, GSSG/GSH ratio and nitrite/nitrate level were estimated using colorimetric assay kits (Cayman chem., Ann Arbor, MI) according to the procedures provided by the manufacturer and were calculated from plotted standard curves.

Assessment of TNFα and IL-6

TNFα and IL-6 levels were measured using rat Enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, USA) according to the manufacturer's instructions at 450 nm and their concentrations were expressed as pg/ml.

Quantification NF-κB activity in PMNs

Isolation of peripheral blood PMNs was performed as previously described by Shih et al. (2010). Briefly, heparinized blood samples were centrifuged at 10,000 × *g* for 15 min. Plasma was removed and the PMNs were isolated using Ficoll- Hypaque and dextran sedimentation. After red blood cells removal by 0.14 mM ammonium chloride solution, the cells were washed twice and resuspended in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 10^6 cells/ml. Samples were used for the extraction.

According to the manufacturer's instructions for nuclear extraction kit (Cayman chem., Ann Arbor, MI), fresh collected PMNs was homogenized in 200 μl of ice-cold hypotonic buffer (10 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 3 μl of 1 M of 10% P-40, complete protease inhibitor cocktail, pH 7.4) for 15 min and was centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant containing the cytoplasmic protein fraction was removed for cell detection, and the remaining nuclear pellet was resuspended in 50 μl of ice-cold extract buffer (hypotonic buffer, 39.8 μl of 5 M NaCl and 5 μl of 10 mM dithiothreitol) for 10 min and was centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant containing the nuclear fraction was used for quantification of NF-κB activity.

NF-κB activity was determined using NF-κB p65 transcription factor assay kit (Cayman chem., Ann Arbor, MI) according to the procedures provided by the manufacturer. Briefly, 2 μg of nuclear extracts were incubated with an oligonucleotide containing the NF-κB consensus site, and then were incubated with a monoclonal and secondary antibody directed against the NF-κB p65 subunit. Reaction was quantified at optical density (OD) 450 nm and was reported as OD450/mg protein. Cayman protein determination kit (Item No: 704002) was used to quantitate protein concentrations in nuclear extracts.

Quantification of apoptosis

Cell death detection ELISA kit (1544675, Roche, Germany) was used to quantitatively detect the cytosolic histone-associated DNA fragmentation, based on the manufacturer's instructions (Siu et al. 2004). Same fractions of selected tissues were excised, rinsed with cold saline and homogenized in ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64, pH 7.4) as discussed previously (Soufi et al. 2008). The homogenates were centrifuged at 1000 rpm for 1 min at 4°C. Cytoplasmic extracts (25 μl) were used as an antigen source in a sandwich ELISA. The change in color was measured at OD 405 nm. The OD reading was then normalized to the total amount of protein in the sample and the data were reported as an apoptotic index (OD_{405}/mg protein) to indicate the level of cell death. Cayman protein determination kit (Item No: 704002) was used to quantitate protein concentrations in cytoplasmic extracts.

Data analysis

Data were expressed as mean ± SE and were analyzed by repeated measure ANOVA (for analysis of the changes in body weights and blood glucose levels) and One-way ANOVA (for other parameters), using SPSS 18 software. When a significant *p*-value was obtained, the Tukey *post-hoc* test was employed to determine the differences between groups. A level of $p < 0.05$ was considered statistically significant.

Results

Body weight and blood glucose

The changes of body weights and blood glucose levels during the experimental period have been presented in Figure 2. While significant weight loss occurred in both diabetic groups ($p < 0.01$ for both); it was lower in DRT group than DC group ($p < 0.05$). There was no significant difference in body weight between NTR and NC groups (Figure 2A).

In comparison to the NC group, blood glucose concentration progressively increased during the protocol period in DC and DTR groups ($p < 0.01$ for both); however, its level in DTR group was significantly lower than DC group ($p < 0.05$). Four-month treatments with resveratrol had not significant effect on the nondiabetic rat blood glucose level statistically (Figure 2B).

Redox state

Figure 3 represents the effect of chronic resveratrol treatment on the plasma levels of oxidative stress markers and blood SOD

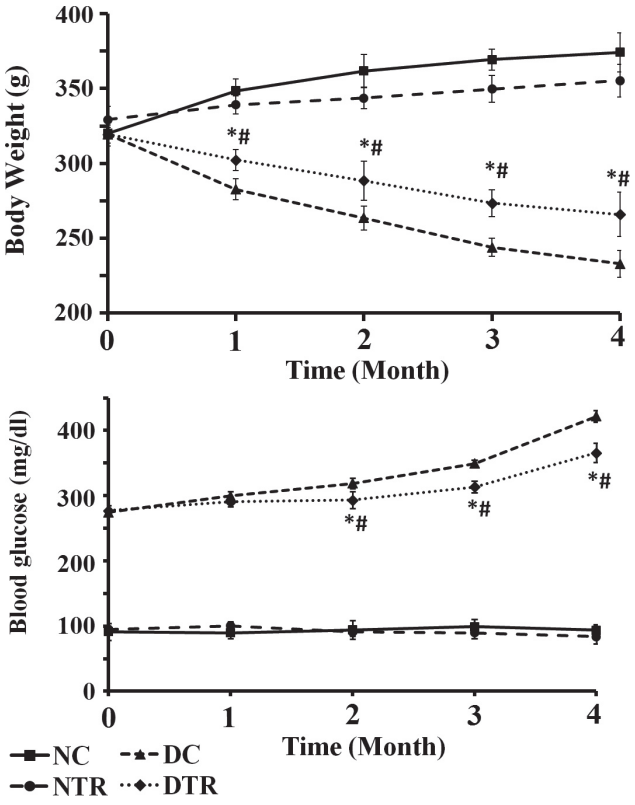

Figure 2. Changes of body weights and blood glucose levels during the experimental period. The values represent mean ± SE of 6 animals *per* group. * $p < 0.01$ *vs.* normal control group (NC); # $p < 0.01$ *vs.* diabetic control group (DC); NTR, normal rats treated with resveratrol; DTR, diabetic rats treated with resveratrol.

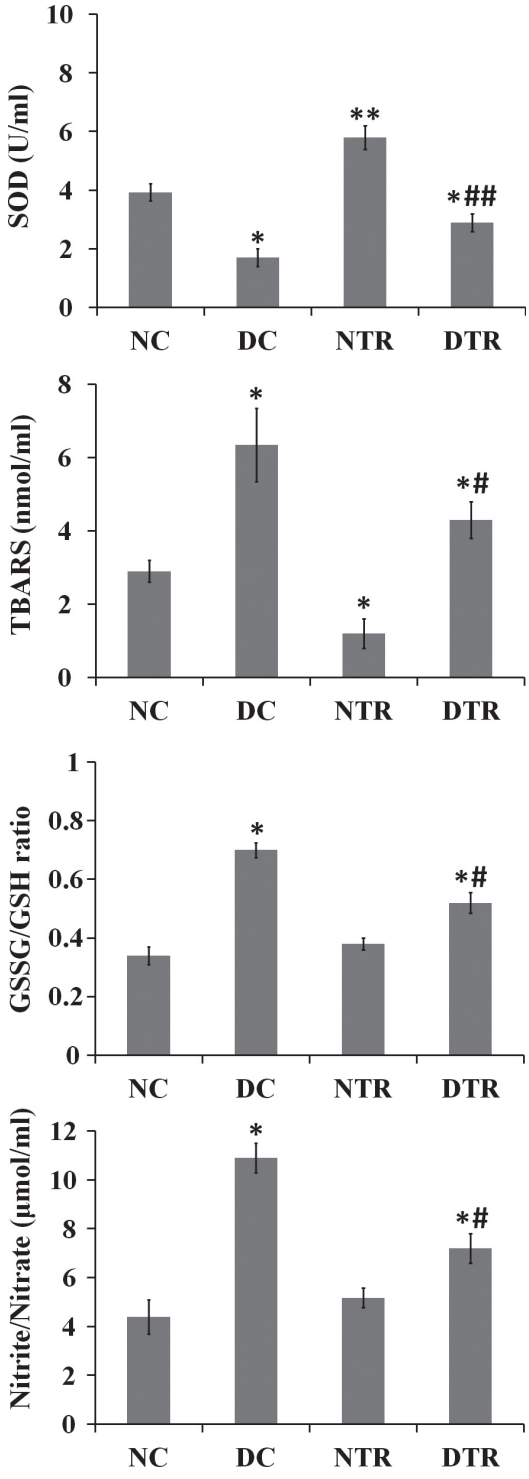

Figure 3. Effect of chronic resveratrol treatment on the plasma levels of TBARS, nitrite/nitrate, GSSG/GSH ratio and the blood activity of superoxide dismutase (SOD). The values represent mean ± SE of 6 animals *per* group. * $p < 0.01$, ** $p < 0.05$ *vs.* normal control group (NC); # $p < 0.01$, ## $p < 0.05$ *vs.* diabetic control group (DC); NTR, normal rats treated with resveratrol; DTR, diabetic rats treated with resveratrol.

activity. In comparison to the normal controls, treatment with resveratrol resulted in enhanced SOD activity ($p < 0.05$) and a decrease in TBARS level in NTR group ($p < 0.01$); while it has no effect on GSSG/GSH ratio and nitrite/nitrate level. We observed reduced SOD activity with concomitant an enhancement in GSSG/GSH ratio, TBARS levels and nitrite/nitrate in both diabetic groups when compared with NC group ($p < 0.01$ for all comparisons). These changes were markedly attenuated by chronic resveratrol administration.

Inflammatory mediators

Figure 4 represents that the plasma concentrations of TNFα and IL-6 were higher in both diabetic groups (DR and DTR) than NC group ($p < 0.01$ for all comparisons); and resveratrol treatment attenuated these changes ($p < 0.01$ for TNFα and $p < 0.05$ for IL-6). In comparison to the normal controls, the levels of TNFα and IL-6 did not significantly change in normal rats treated with resveratrol.

Figure 4C depicts that the NF-κB activity significantly increased in the PMNs of DC and DTR groups as compared with normal groups ($p < 0.01$ for DC group and $p < 0.05$ for DTR group comparisons). Treatment with resveratrol reduced these enhancements statistically ($p < 0.05$). There was no significant difference in NF-κB activity between NTR and NC groups.

Apoptosis rate

The levels of apoptosis rate increased in the PMNs, kidney, liver, heart, aorta, and sciatic nerve of DR and DTR groups ($p < 0.01$ for all) as compared with normal controls (Figure 5). Treatment with resveratrol reduced these enhancements, except in the aorta ($p < 0.01$ for PMNs and $p < 0.05$ for others). There was no significant difference in apoptosis rate between NTR and NC groups.

Discussion

Insufficient secretion or action of insulin causes hyperglycemia, mainly *via* an enhanced release of glucose by the liver and reduced utilization of glucose in peripheral tissues. In this situation, the body has to provide itself energy by degradation of proteins and lipids from their reservoirs, which ultimately accounts for weight loss (Palsamy and Subramanian 2008; Roghani and Baluchnejadmojarad 2010). Treatment with resveratrol attenuated weight loss process in our diabetic rats, suggesting possible improvement in energy metabolism. This observation is in agreement with pervious published literatures (Palsamy and Subramanian 2008, 2010).

During normal condition, moderate amounts of reactive oxygen species (ROS) including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), which mainly are produced by mitochondria and NAD(PH) oxidase, contribute in some physiological process. Dismutation of $O_2^{\bullet -}$ (the most abundant reactive oxygen radical producing in the cells) to H_2O_2 by SOD, is the first step in ROS detoxification. Then, H_2O_2 is metabolized into water by the activities of catalase and glutathione peroxidase. Moreover, GSH, a co-substrate for glutathione peroxidase activity, is another major intra- and extracellular antioxidant molecule and acts as a direct free radical scavenger by conversion to GSSG. GSSG to GSH ratio is a good marker for estimation of redox state (Palsamy and Subramanian 2010). Overproduction of ROS or reduction in the ability of the endogenous antioxidants to neutralize ROS, results in oxidative stress which in turn can lead to damages of

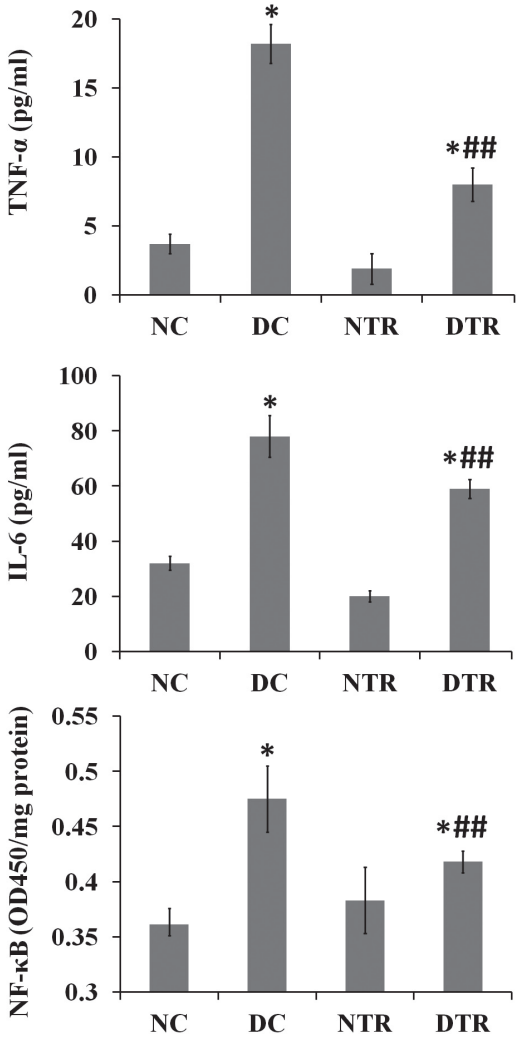

Figure 4. Role of long-term resveratrol administration on the plasma concentration of TNFα and IL-6 as well as NF-κB activity in polymorphonuclear cells. For abbreviations and symbols, see Figure 3.

lipids, proteins and DNA. This situation is traceable by measurements of lipid peroxidation, protein oxidation and DNA fragmentation or cell death. Also, nitric oxide ($NO^{\bullet}$), another contributor to oxidative stress, can react with $O_2^{\bullet -}$ to form a potent oxidizing agent, peroxynitrite ($ONOO^-$), and account for more cellular damage (Rains and Jain 2011). Due to very low concentration and the short half-life of $NO^{\bullet}$, its stable metabolites such as nitrite (NO_2^-) and nitrate (NO_3^-) are determined as suitable indices for estimation of $NO^{\bullet}$ (Pitocco et al. 2010).

It has been reported that chronic hyperglycemia results in oxidative stress either by the direct generation of ROS or by altering the redox balance *via* increased polyol pathway flux (which reduces NADPH reservoirs and reduces conversion of GSSG to GSH), increased formation of advanced glycation end products, activation of protein kinase C and overproduction of superoxide by the mitochondrial electron transport chain (Rains and Jain 2011). In this context, our data completely are in agreement with the previous studies, in which our diabetic control rats experienced chronic hyperglycemia with enhancements in oxidative stress markers (including lipid peroxidation, nitrite/nitrate and GSSG to GSH ratio) with concomitant reduction in SOD activity.

Many of the above mentioned hyperglycemia-induced pathways converge to activate NF-κB, a proinflammatory master switch, which activates proinflammatory cytokines gene expressions (Palsamy and Subramanian 2010). Chronic inflammation in diabetes is mainly based on the elevated levels of proinflammatory cytokines such as TNFα and IL-6 (van den Oever et al. 2010). These cytokines promote inflammation through local recruitment of monocytes within tissues leading to resistance to insulin, endothelial dysfunction and atherothrombosis (van den Oever et al. 2010). It should be noticed that TNFα-activated pathway in turn activates NF-κB and apoptotic processes and exacerbates diabetes-related complications (King 2008; van den Oever et al. 2010). On the other hand, NF-κB also increases inducible nitric oxide synthase expression leading to enhanced production of $NO^{\bullet}$ (Palsamy and Subramanian 2010).

In the context of inflammation and its-related apoptosis, our data also are in line with previous studies in which TNF-α and IL-6 plasma concentration, NF-κB activity of the PMNs and tissues apoptosis rates of DC and DTR groups were significantly higher than normal controls.

Among beneficial antidiabetic actions of resveratrol, its antioxidant effect seems to be best documented. A variety of animals and human studies have shown that short-term resveratrol administration for diabetic subjects (up to two months), could enhance the antioxidant defense, reduce lipid and protein oxidation and decrease apoptosis rate (Kumar et al. 2007; Palsamy and Subramanian 2010; Singh et al. 2011). The antioxidant property of resveratrol may be performed directly by modulating the antioxidants gene expression and its free radical quenching action or indirectly by reducing of hyperglycemia (Roghani and Baluchnejadmojarad 2010; Akar et al. 2011; Yun et al. 2011). Moreover, it has been documented that short-term treatment with resveratrol in diabetic subjects has inhibited the activation of NF-κB and the production of $NO^{\bullet}$, TNFα and IL-6 at transcriptional or post-transcriptional levels (Lee et al. 2009; Zhang et al. 2010). Our data are in accordance with the results of literatures obtaining from short-term resveratrol administration in diabetic subjects in which, 4-months oral resveratrol intake increased blood SOD activity, reduced plasma oxidative stress markers (GSSG/GSH ratio, lipid peroxidation and nitrite/nitrate) and inflammatory mediators (TNFα, IL-6 and NF-κB) and finally decreased apoptosis rates in PMNs and some other tissues.

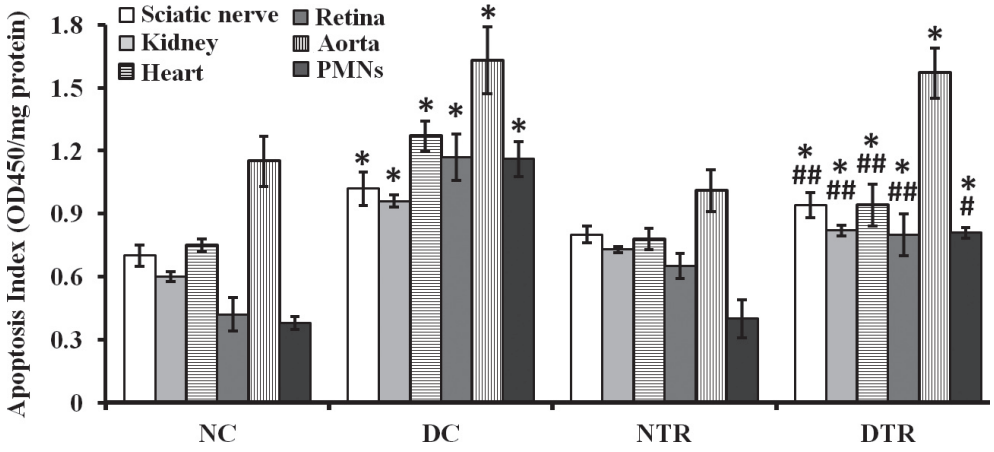

Figure 5. Effect of four months resveratrol administration on apoptosis rate. For abbreviations and symbols, see Figure 3.

In conclusion, our results depict that chronic resveratrol administration has an anti-hyperglycemic effect. Moreover, it reduces antioxidant machinery impairment and reverts back the levels of inflammatory mediators and the rate of apoptosis in diabetic rats. Given the safety and low adverse effect of resveratrol, these beneficial antidiabetic observations suggest that treatment with resveratrol may be considered as a therapeutic approach to reduce diabetic-related complications.

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