Long-term Resveratrol Administration Reduces Renal Oxidative Stress and Apoptosis Rate In Experimental Model of Type 2 Diabetes.

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Abstract: The present study was designed to evaluate whether long-term resveratrol administration has beneficial effects on renal oxidative stress 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. RESULTS: Four-month oral resveratrol prescriptions (5 mg/kg/day) significantly attenuated the enhancement of blood glucose, glycosylated hemoglobin, urea, and creatinine and 8-isoprostane levels in diabetic rats. Moreover, resveratrol administration to diabetic rats improved the reduced levels of glutathione, total antioxidant capacity and the antioxidant enzymes activities (superoxide dismutase, glutathione peroxidase and catalase). The apoptosis rate significantly increased in the renal of diabetic groups as compared with normal groups. Treatment with resveratrol reduced this enhancement statistically. These results suggest that chronic resveratrol administration is safe and effective and also may be considered as a therapeutic compound in diabetes.
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1. Introduction

Diabetes mellitus, a chronic and progressive metabolic disorder, is a challenging public health problem and nowadays, diabetic nephropathy and its-related renal failure are one of the most important contributing mortality factors in developing countries (Luis-Rodríguez *et al.*, 2012; Balakumar *et al.* 2009). Although the pathogenesis of diabetic nephropathy is multifactorial hyperglycemia-induced oxidative stress plays a crucial role (Palsamy and Subramanian, 2011; Kitada *et al.,* 2011; Chang *et al.,* 2011).

Diabetes-related hyperglycemia is resulted from insufficient secretion or action of endogenous insulin and induces oxidative stress via enhancement of glucose oxidation, advanced glycation end products, protein kinase C, hexosamine and polyol pathways fluxes and pro-inflammatory cytokines (Rains and Jain, 2011). It widely has been accepted that oxidative stress, an imbalance 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). 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 patient's health problems.

Resveratrol (trans-3, 5, 4'-trihydroxystilbene, discovered in 1940s) is a polyphenolic phytoalexin present in different plants such as grapes, peanuts and berries (Cottart *et al.,* 2010). Numerous *in vivo* and *in vitro* studies have been reported that resveratrol has many 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 in the pathogenesis of diabetes, in the recent years, numerous investigations have focused on the role of resveratrol in prevention or treatment of diabetic nephropathy (Palsamy and Subramanian, 2011; Kitada *et al.,* 2011; Chang *et al.,* 2011; Chen *et al.,* 2011). In this regard, it has been reported that short-term treatment of resveratrol (3-8 weeks) has been beneficial renoprotective effects, mainly via reducing Oxidative stress and enhancement of

antioxidants enzymes activities (Palsamy and Subramanian, 2011; Kitada *et al.,* 2011; Chang *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 tolerated. 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 administration of resveratrol can attenuate oxidative stress and apoptosis rate in the kidney of streptozotocin (STZ)-nicotinamide model of diabetic rats

2. Material and Methods

2.1. 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 of 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 prescription of nicotinamide (110 mg/kg; i.p.) in 12 h fasted rats. Citrate buffer were injected alone in control rats. Nicotinamide preserves the pancreatic β-cells (up to 40 %) from STZ cytotoxicity and produces NIDDM similar to human NIDDM (Masiello et al., 1998). To prevent from the fatal hypoglycemic effect of pancreatic insulin release, 10 % glucose solution, were 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 250 mg/dl were included to the protocol as diabetic rats. Resveratrol treatment (5 mg/kg) was carried out orally in aqueous solution for four months. The dosage was regulated every week. At the end of experimental period, fasted rats were anesthetized with ketamine (80 mg/kg) and blood samples (5 ml) were collected from each rat for biochemical measurements. Then, the rats were killed by cervical decapitation, the kidneys quickly removed, weighted and washed in cold saline and freezed at -80 °C. All manipulations take placed in morning. All above chemicals (except resveratrol) were purchased from Sigma (Sigma, St. Louis, MO, USA). Resveratrol was obtained from Cayman chemicals (Cayman chem., Ann Arbor, MI, USA).

Biochemical measurements

Blood glucose, glycosylated hemoglobin (HbA1c), urea and creatinine were measured spectrophotometrically by the zistshimi lab Kits (Roghani and Baluchnejadmojarad, 2010).

2.2. Oxidative stress measurements

Evaluation of the kidney was carried out by measurements of antioxidant enzymes activities (superoxide dismutase; SOD, glutathione peroxidase; GPx, and catalase; CAT), the levels of glutathione (GSH) and 8-Isoprostane colorometrically, using the Cayman chemicals assay kits (Cayman chem., Ann Arbor, MI, USA) in accordance to manufacturer's instructions. Tissue preparation was performed by homogenizing of the same portion of the right kidneys (50 mg) in ice-cold buffer containing 10 mM NaCl, 2 mM MgCl2, 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. After centrifugation at 14000 g for 10 min at 4°C, the supernatant containing the cytoplasmic protein fraction was used for determination of cell death detection and oxidative stress markers. Cayman protein determination kit (Item No: 704002) was used to quantitate protein concentrations.

2.3. 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. Renal cytoplasmic extracts (25 µl) were used as an antigen source in a sandwich ELISA. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). 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.

2.4. Data analysis

Data were expressed as mean ± SD and were analyzed by One-way ANOVA, 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 significance.

3. Results

The changes in biochemical measurements and renal and body weights have been presented in Table 1. Although significant weight loss occurred in both diabetic groups, but this weight loss in DRT group was markedly lower than DC group ($p < 0.01$ for all

comparisons). We did not see significant changes in body weights between NTR and NC groups.

In comparison to the NC group, blood glucose concentration increased in DC and DRT groups ($p < 0.01$ for both); however, its level in DRT group was significantly lower than DC group ($p < 0.01$). Four-month treatments with resveratrol have not significant effect on the nondiabetic rat's blood glucose level statistically.

The levels of urea, creatinine and HbA1c were higher in both diabetic groups ($p < 0.01$ for all comparisons) when compared with NC group (table 1). Four-month treatments with resveratrol statistically reduced HbA1c, urea and creatinine levels in DRT group when compared with DC group ($p < 0.01$ for all). Table 1: Effect of 4-month oral resveratrol administration on body weight, renal weight and blood biochemistry.

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Groups / Indices	Normal Control (NC)	Diabetic Control (DC)	Normal Treated with Resveratrol (NTR)	Diabetic Treated with Resveratrol (DTR)
Body weight (g)	376.41 ± 5.61	229.91 ± 4.76*	352.63 ± 6.08	272.59 ± 8.19*#
Glucose (mg/dl)	104.29 ± 3.44	421.58 ± 5.01*	97.91 ± 4.96	356.17 ± 5.29*#
HbA1c (% Hb)	7.46 ± 0.12	16.61 ± 0.20*	8.11 ± 0.17	12.17 ± 0.14*#
Creatinine (mg/dl)	0.69 ± 0.02	1.73 ± 0.03*	0.74 ± 0.03	1.23 ± 0.03*#
Urea (mg/dl)	16.50 ± 1.2	59.44 ± 2.9*	19.16 ± 2.6	35.24 ± 1.4*#

The values represent mean ± SD of 6 animals per group. * $p < 0.01$ versus normal control group (NC). # $p < 0.01$ versus diabetic control group (DC). SOD: superoxide dismutase, GPX: glutathione peroxidase, CAT: catalase, GSH: glutathione, ALT: alanine transaminase, AST: aspartate transaminase, and ALP: alkaline phosphatase.

Table 2 also represents the effects of chronic resveratrol treatment on renal oxidative stress. In comparison to the NC group, treatment with resveratrol enhanced SOD activity and reduced 8-Isoprostane level in NTR group, while it has no effect on GPx and CAT activities and the level of GSH ($p < 0.01$ for all comparisons). In both diabetic groups, the activities of all antioxidant enzymes (SOD, GPx and CAT) and the levels of GSH were decreased, and the levels of 8-Isoprostane were increased when compared with NC group; Treatment with resveratrol was markedly attenuated these changes ($p < 0.01$ for all comparisons).

Figure 1 depicts that the apoptosis rate significantly increased in the renal 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 this enhancement statistically ($p < 0.05$). There was no significant difference in apoptosis rate between NTR and NC groups.

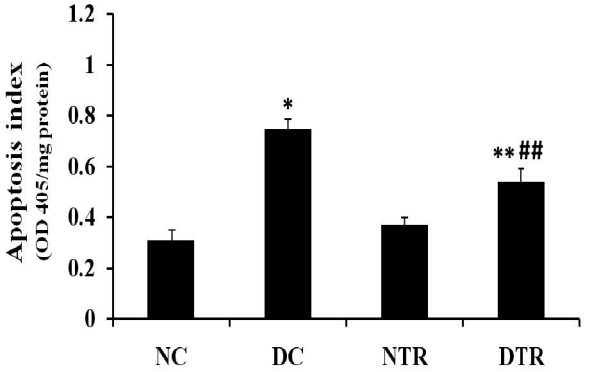

Figure 1: Effect of four months resveratrol administration on the renal apoptosis rate in diabetic rats. The values represent mean ± SD of 6 animals per group. * $p < 0.01$ and ** $p < 0.05$ versus normal control group (NC). ## $p < 0.05$ versus diabetic control group (DC). NTR: normal rats treated with resveratrol, and DTR: diabetic rats treated with resveratrol.

Table 2: Effects of chronic resveratrol treatment on renal oxidative stress.

Groups / Indices	Normal Control (NC)	Diabetic Control (DC)	Normal Treated with Resveratrol (NTR)	Diabetic Treated with Resveratrol (DTR)
8-Isoprostane (pg/ml)	877.2 ± 12.38	1513.6 ± 9.30*	833.4 ± 11.01*	1157.9 ± 16.1*#
GSH (µmol/ml)	23.82 ± 1.17	11.61 ± 1.03*	19.15 ± 1.19	16.13 ± 0.88*#
SOD (U/g Hb)	249.26 ± 11.8	146.19 ± 19.6*	332.21 ± 15.3*	211.18 ± 10.6*#
GPX (U/g Hb)	357.21 ± 21.3	189.34 ± 18.6*	366.91 ± 11.9	261.77 ± 9.44*#
CAT (nmol/min/ml)	3.21 ± 0.16	1.03 ± 0.07*	2.66 ± 0.11	1.79 ± 0.14*#

The values represent mean ± SD of 6 animals per group. * $p < 0.01$ versus normal control group (NC). # $p < 0.01$ versus diabetic control group (DC). SOD: superoxide dismutase, GPX: glutathione peroxidase, CAT: catalase, GSH: glutathione

4. 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 accumulation of protein and lipid by/end products

(such as urea, creatinine, free fatty acids, triglyceride and cholesterol), reduction of plasma total proteins and finally weight loss (Palsamy and Subramanian, 2008; Roghani and Baluchnejadmojarad, 2010). It should be noticed that, liver or kidney malfunction induced by chronic hyperglycemia, in turn, reduces plasma proteins, enhances proteinurea, accumulates urea and creatinine and accelerates weight loss (Palsamy and Subramanian, 2008). Our results showed that blood glucose, urea and creatinine concentrations markedly elevated after four months of diabetes and concomitantly body weight decreased during this period. Alleviation of these disturbances with resveratrol treatment, suggest that chronic administration of resveratrol has been beneficial antidiabetic effects.

Oxidation of glucose is one of the mechanisms involved in pathogenesis of diabetes complications (Maritim *et al.,* 2003). Glucose oxidation enhances glycation of proteins such as hemoglobin (and produces HbA1c) and antioxidant enzymes which in turn, can reduce their activities for detoxification of reactive oxygen/nitrogen-free radicals and lead to lipids, proteins and DNA peroxidation and finally programmed cell death (Rains and Jain, 2011). The concentration of HbA1c is considered as a good marker for diagnosis and prognosis of diabetes complications. Although the anti-hyperglycemic effect of chronic resveratrol administration was small in this study, but it reduced HbA1c approximately 4.44%. It has been reported that, there is strong correlation between HbA1c and risk of diabetic retinopathy, nephropathy and neuropathy (Howlett and Ashwell, 2008) and reduction of HbA1c by only 1 unit (8% to 7%) can reduce the risk of retinopathy by over 30% (Kowluru and Chan, 2007).

8-Isoprostane (8-*iso* prostaglandin $F_{2\alpha}$), a member of eicosanoids family producing by the oxidation of tissue phospholipids by oxygen radicals, has been proposed as a marker of antioxidant deficiency and oxidative stress (Morrow *et al.,* 1995). It has been shown that plasma concentration of 8-isoprostane increases with diabetes-induced lipid peroxidation and oxidative stress (Ndisang *et al.,* 2010; Salim *et al.,* 2010). Reducing of 8-isoprostane concentrations in normal and diabetic rats after the 4-month period of resveratrol treatment shows that resveratrol has a strong antioxidant effect and attenuates oxidative stress.

Dismutation of superoxide radicals (the most abundant reactive oxygen radical producing in the cells) to hydrogen peroxide is the first step in detoxification of reactive oxygen/nitrogen species. Then, hydrogen peroxide is metabolized into water by the activities of CAT and GPx. Moreover, GSH, a co-substrate for GPx activity, is a major intracellular antioxidant molecule and acts as a direct free radical scavenger (Palsamy and Subramanian, 2010). Antioxidant machinery impairment duo to antioxidant enzymes and other proteins glycations have been previously reported (Palsamy and Subramanian, 2010). On the other hand, Davi et al. have shown that following the activation of polyol pathway and consumption of NADH, GSH availability for efficient function of GPx reduces in diabetes mellitus (Davi *et al.,* 2005). Our data shown that after four months of diabetic state, GSH concentrations as well as SOD, GPx and CAT activities decreased in both diabetic groups (table 2). Chronic resveratrol administration not only attenuated observed antioxidant machinery impairments in diabetic rats, but also it increased SOD and CAT activities in normal rats. These results are in line with obtained results from studies administrating resveratrol for shorter time (Palsamy and Subramanian, 2011; Kitada *et al.,* 2011; Chang *et al.,* 2011). This observation suggests that the antioxidant properties of resveratrol may be accomplished directly or through reducing blood glucose. Schmatz et al. have previously proposed that antioxidant effect of resveratrol do not depend on its hypoglycemic property (Schmatz *et al.* 2009).

Many of above mentioned hyperglycemia-induced pathways converge to elevate NF-κB, a proinflammatory master switch, which activates proinflammatory cytokines gene expressions and apoptosis cascade (Kern 2007, Palsamy and Subramanian, 2010). Our data also are in line with previous studies, in which the renal apoptosis rates in DC and DTR groups were significantly higher than normal controls (Barber *et al.* 2011, Kern 2007). Reducing renal apoptosis rates after 4-month resveratrol intake is another certification to support the beneficial effect of resveratrol in preventing diabetes complications.

In conclusion, our results depict that chronic treatment with resveratrol has an effective anti-hyperglycemic effect, leading to reduction in HbA1c level in diabetic rats. Moreover, it reduced renal antioxidant machinery impairment, apoptosis rate and some diabetic complication markers, including blood urea and creatinine concentrations. It is possible that resveratrol improves cellular functions through reducing oxidative stress which in turn, reduces diabetes- induced hyperglycemia and its related complications. Similar to the obtained results previously from short-term administration of resveratrol, our results suggest that, chronic resveratrol administration is safe and effective and also may be considered as a therapeutic compound in diabetes.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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