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P. Palsamy, S. Subramanian. Resveratrol protects diabetic kidney by attenuating hyperglycemia mediated-oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2011, 10.1016/j.bbadis.2011.03.008 . hal-00694734

**HAL Id: hal-00694734**

**<https://hal.science/hal-00694734>**

Submitted on 6 May 2012

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## Accepted Manuscript

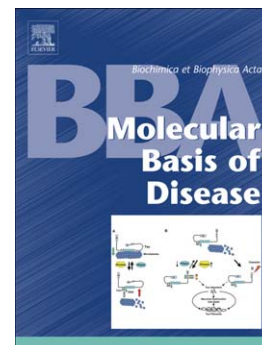
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PII: S0925-4439(11)00063-9  
DOI: doi: [10.1016/j.bbadis.2011.03.008](https://doi.org/10.1016/j.bbadis.2011.03.008)  
Reference: BBADIS 63264

To appear in: *BBA - Molecular Basis of Disease*

Received date: 18 December 2010  
Revised date: 10 March 2011  
Accepted date: 15 March 2011



Please cite this article as: P. Palsamy, S. Subramanian, Resveratrol protects diabetic kidney by attenuating hyperglycemia mediated-oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling, *BBA - Molecular Basis of Disease* (2011), doi: [10.1016/j.bbadis.2011.03.008](https://doi.org/10.1016/j.bbadis.2011.03.008)

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**Original Article**

**Resveratrol protects diabetic kidney by attenuating hyperglycemia mediated-oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling**

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**Abstract**

Hyperglycemia-mediated oxidative stress plays a crucial role in the progression of diabetic nephropathy. Hence, the present study was hypothesized to explore the renoprotective nature of resveratrol by assessing markers of oxidative stress, proinflammatory cytokines and antioxidant competence in streptozotocin-nicotinamide-induced diabetic rats. Oral administration of resveratrol to diabetic rats showed a significant normalization on the levels of creatinine clearance, plasma adiponectin, C-peptide and renal superoxide anion, hydroxyl radical, nitric oxide, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and NF- $\kappa$ B p65 subunit and activities of renal aspartate transaminase, alanine transaminase and alkaline phosphatase in comparison with diabetic rats. The altered activities of renal aldose reductase, sorbitol dehydrogenase and glyoxalase-I and elevated level of serum advanced glycation end products in diabetic rats were also reverted back to near normalcy. Further, resveratrol treatment revealed a significant improvement in superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase activities and vitamins C and E, and reduced glutathione levels, with a significant decline in lipid peroxides, hydroperoxides and protein carbonyls levels in diabetic kidneys. Similarly, mRNA and protein analyzes substantiated that resveratrol treatment notably normalizes the renal expression of Nrf2/Keap1 and its downstream regulatory proteins in the diabetic group of rats. Histological and ultrastructural observations also evidenced that resveratrol effectively protects the kidneys from hyperglycemia-mediated oxidative damage. These findings demonstrated the renoprotective nature of resveratrol by attenuating markers of oxidative stress in renal tissues of diabetic rats.

*Keywords:* Advanced glycation end products; Diabetic kidney; Nrf2/Keap1 signaling; Oxidative stress; Proinflammatory cytokines; Resveratrol

*Abbreviations:* AGEs, advanced glycation end products; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; Ccr, Creatinine clearance; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid;  $\gamma$ -GCS,  $\gamma$ -glutamylcysteine synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S- transferase; HO-1, heme oxygenase -1; Keap1, Kelch like-ECH-associated protein 1; LSD, least significant difference; NO, nitric oxide; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Pcr, plasma creatinine; PMSF, phenylmethylsulfonyl-fluoride; SOD, superoxide dismutase; Ucr, urinary creatinine.

## 1. Introduction

Diabetes mellitus is a heterogeneous metabolic disorder essentially characterized by insufficiency of insulin secretion and insulin receptor or postreceptor events with derangement in carbohydrate, protein and lipid metabolism resulting in chronic hyperglycemia. Hyperglycemia is a well distinguished pathogenetic factor of long-term complications in diabetes mellitus not only engenders excessive free radicals but also attenuates antioxidative machineries through glycation of the antioxidant enzymes. Hence, oxidative stress has been considered to be a general pathogenic factor of diabetic complications including nephropathy [1]. Diabetic nephropathy, an imperative complication of diabetes mellitus, is characterized by the protuberance of the glomerular mesangium due to amassing of extracellular matrix proteins synthesized by the mesangial cells with basement membrane thickening, glomerular and tubular hypertrophy, glomerulosclerosis and tubulointerstitial fibrosis [2]. As hyperglycemia-mediated oxidative stress plays a central role in the development and progression of diabetic nephropathy, glycemic control remains the focal target in the treatment of diabetic nephropathy.

Agents with significant antioxidant potentials have been reported to elicit protection on diabetic kidney damage. The antioxidants such as taurine, melatonin, vitamin C and vitamin E are reported to reduce the renal complications like glomerular hypertrophy, albuminuria, glomerular collagen and renal protein kinase C activity in experimental diabetes [3, 4]. Glyclazide, the second generation sulfonylurea, has also been shown to possess a notable antioxidant potential due to its azabicyclo-octyl ring independent of glycemic control by scavenging free radicals, reducing the expression of NAD(P)H oxidase, enhancing renal expression of Mn-superoxide dismutase and endothelial nitric oxide synthase, suppressing glomerular macrophage migration thereby ameliorating glomerular matrix expansion and albuminuria [5]. Hence, it is recommended that therapy with antioxidants may signify a

useful pharmacologic overture to the management of diabetes. Therefore, the present study was aimed to assess the antioxidant defensive as well as renal tissue protective nature of resveratrol (3,5,4'-trihydroxystilbene) in streptozotocin-nicotinamide-induced diabetic rats.

Resveratrol is a polyphenolic phytoalexin that occurs naturally in many plant species, including grapevines and berries and exhibits surfeit of pharmacologic health benefits including antioxidant [6], antimutagenic [7], anti-inflammatory [8], estrogenic [9], antiplatelet [10], anticancer [11] and cardioprotective properties [12]. There has been copious epidemiologic and clinical evidence confirming that resveratrol may act as an antioxidant through enhancing hydrogen peroxide tolerance and inhibiting cyclooxygenase-2 activity. Recently, resveratrol has been reported to possess antihyperglycemic effect in experimental diabetes [13], which is mediated by modulating the activities of key carbohydrate metabolizing enzymes in the hepatic and renal tissues of experimental diabetic rats [14-15]. More recently, we have reported the pancreatic  $\beta$ -cell [16] as well as hepatocytes protective nature of resveratrol from oxidative damage in streptozotocin-nicotinamide-induced diabetic rats [17]. Moreover, very limited studies have demonstrated the effect of resveratrol on diabetic kidney by assessing the changes in phosphorylation of histone H3, levels of the oxidative markers such as malondialdehyde and glutathione and antioxidant enzymes such as superoxide dismutase and catalase in the renal tissues of diabetic rats [18, 19] and none of them have explored the protective nature of resveratrol on renal tissue ultrastructure during hyperglycemia- as well as proinflammatory cytokines-mediated oxidative damage in streptozotocin-nicotinamide-induced diabetic rats. Hence, the present study was aimed to investigate the ameliorative potential of resveratrol on proinflammatory cytokines, hyperglycemia-mediated oxidative stress and renal dysfunction in streptozotocin-nicotinamide-induced diabetic rats and the efficacy of resveratrol was compared with glyclazide, an oral antihyperglycemic drug with antioxidant potential.

## 2. Methods and Materials

### 2.1. Chemicals

Resveratrol, streptozotocin, nicotinamide, epinephrine, thiobarbituric acid, reduced glutathione (GSH), vitamin E and sodium azide were procured from Sigma Chemicals Co., St. Louis, MO, stored at 2-4°C and protected from sunlight. All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

### 2.2. Experimental animals

Animal experiments were premeditated and executed in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (Approval No.01/036/07). Six-week-old, male, Wistar rats weighing 160–180 g, procured from Tamilnadu Veterinary and Animal Sciences University, Chennai, India, were housed in clean, sterile, polypropylene cages under standard vivarium conditions (12 h light/dark cycle) with *ad libitum* access to water and standard rat chow (Hindustan Lever Ltd., Bangalore, India) with a composition of 5% fat, 21% protein, 55% nitrogen-free extract and 4% fiber (w/w) with adequate mineral and vitamin levels for the animals. The animals were acclimatized to the laboratory conditions for 2 week prior to the inception of experiments.

### 2.3. Induction of experimental diabetes

Experimental diabetes was induced in 12 h fasted rats by single i.p. injection of streptozotocin (50 mg/kg body weight) dissolved in 100 mM cold citrate buffer, pH 4.5 [20], 15 min after the intraperitoneal administration of nicotinamide (110 mg/kg body weight) [21]. Since streptozotocin is capable of inducing fatal hypoglycemia as a result of massive



pancreatic insulin release, the rats were supplied with 10% glucose solution after 6 h of streptozotocin administration for the next 24 h to prevent hypoglycemia [22]. After a week in time for the development and aggravation of diabetes, rats with moderate diabetes (i.e. blood glucose concentration  $\geq 14$  mM) were selected for the experiment.

#### 2.4. *Experimental design*

The experimental animals were divided into five groups, each group comprising of a minimum of six rats as detailed follows. Group 1 served as control rats; Group 2 served as control rats daily administered with resveratrol (5 mg/kg body weight) in aqueous suspension orally for 30 days; Group 3 served as streptozotocin-nicotinamide-induced diabetic rats; Group 4 served as diabetic rats daily administered with resveratrol (5 mg/kg body weight) in aqueous suspension orally for 30 days, and Group 5 served as diabetic rats daily administered with glyclazide (5 mg/kg body weight) in aqueous suspension orally for 30 days.

During the experimental period, body weight, blood glucose, food and water consumption and physical examinations were determined at regular intervals. The dosage was adjusted every week according to any change in body weight to maintain similar dose per kg body weight of rat over the entire period of study for each group. At the end of the treatment period, the rats were fasted overnight, anaesthetized with ketamine (80 mg/kg body weight; i.p.) and killed by cervical decapitation. The blood was collected with or without anticoagulant for plasma or serum separation, respectively. Fasting blood glucose, HbA1c and plasma insulin levels were determined to confirm the antihyperglycemic property of resveratrol [14, 15].

#### 2.5. *Preparation of kidney tissue homogenate*

Kidney tissues from control and experimental groups of rats were excised, rinsed with ice-cold saline and homogenized in 100 mM Tris-HCl buffer (pH 7.4) using Teflon homogenizer and centrifuged at 12,000g for 30 min at 4°C. The supernatant was pooled and used for the estimations. The protein content in the tissue homogenate was also estimated [23].

#### 2.6. *Determination of intraperitoneal insulin tolerance*

At the end of the experimental period, fasting blood samples were withdrawn from the control and experimental groups of rats. Four more blood samples were collected at 30, 60, 90 and 120 min intervals after the intraperitoneal administration of a bolus of insulin (2 unit/kg body weight). All the blood samples were collected with ethylenediaminetetraacetic acid (EDTA) for the determination of glucose by using glucose oxidase peroxidase diagnostic enzyme kit (Span Diagnostic Chemicals, Surat, India).

#### 2.7. *Assessment of renal dysfunction*

The levels of plasma and urinary creatinine (Pcr and Ucr) were determined by alkaline picrate method using creatinine assay kit (Span Diagnostics Ltd, Surat, India). 24 h urine samples were collected using metabolic cages. Creatinine clearance (Ccr) was computed using the following formula:  $Ccr (\mu\text{L}/\text{min}) = (\text{Ucr}/\text{Pcr}) \times \text{urine volume } (\mu\text{L}/\text{min})$ . The activities of pathophysiological enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were also assayed in the kidney tissue homogenate of control and experimental groups of rats [24, 25].

#### 2.8. *Determination of plasma adiponectin and C-peptide*

The concentration of plasma adiponectin was determined by ELISA (Biosource, Camarillo, CA) and plasma C-peptide assay was performed by using an ELISA kit supplied by Linco Research Inc., St Charles, MO. Both the analyses were done according to the manufacturer's instructions.

#### 2.9. Assay of renal polyol pathway enzymes

The activities of aldose reductase [26], sorbitol dehydrogenase [27] and glyoxalase-I [28] were determined in the kidney tissue homogenate of control and experimental groups of rats. The concentration of serum advanced glycation end products (AGEs) was measured by ELISA (Abcam, Cambridge, UK) as per the manufacturer's instructions.

#### 2.10. Assay of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and NF- $\kappa$ B p65 subunit

The levels of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in kidney tissues of control and experimental groups of rats were determined by specific ELISA kits according to the manufacturer's instructions (Biosource, Camarillo, CA). The concentration of proinflammatory cytokines was determined spectrophotometrically at 450 nm. Standard plots were constructed by using standard cytokines and the concentrations for unknown samples were calculated from the standard plot. The nuclear level of p65 may correlate positively with the activation of NF- $\kappa$ B pathway. The NF- $\kappa$ B/p65 ActivELISA (Imgenex, San Diego, CA) kit was used to quantify NF- $\kappa$ B free p65 in the nuclear fraction of kidney tissue homogenate. The analysis was done according to the manufacturer's instructions.

#### 2.11. Assay of renal superoxide radical, hydroxyl radical and total nitric oxide (NO)

The generation of superoxide radical in the kidney tissue homogenate of control and experimental groups of rats was measured spectrophotometrically at 510 nm using the

cytochrome *C* method and the superoxide radicals generated was expressed as absorbance at 550 nm/15 min [29]. The generation of hydroxyl radical in the kidney tissue of control and experimental groups of rats was measured spectrophotometrically at 510 nm and was expressed as absorbance at 510 nm/30 min [30]. The renal NO level was indirectly measured by determining the nitrite level using a colorimetric method based on the Griess reaction [31].

#### 2.12. *Measurement of hyperglycemia-mediated oxidative stress*

The levels of non-enzymatic antioxidants such as vitamin C [32], vitamin E [33] and GSH [34] were estimated in the kidney tissue homogenate of control and experimental groups of rats. Further, the levels of lipid peroxides [35], hydroperoxides [36] and protein carbonyls [37] were determined in the kidney tissue homogenate of control and experimental groups of rats. The activities of enzymatic antioxidants such as superoxide dismutase (SOD) [38], catalase [39], glutathione peroxidase (GPx) [40], glutathione-S- transferase (GST) [41] and glutathione reductase (GR) [42] were assayed in the kidney tissue homogenate of control and experimental group of rats.

#### 2.13. *Real-time qPCR analysis for Nrf2, Keap1, $\gamma$ Gcsh, and Gstm3*

Total RNA was extracted from the renal tissues from control and experimental groups of rats by TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA), according to the manufacturers protocol and purified by Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Purified total RNA was reverse transcribed into single strand cDNAs, which were successively analyzed by real-time qPCR using the SYBR GREEN PCR master mix (Applied Biosystems, Foster City, CA). The amplification protocol comprised of 1 cycle at 95°C for 3 min followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and then 72°C for 30 s. Primer sequences were designed using the Primer Express 2.0 software ensuing the instructions of

Applied Biosystems' for optimal primer design and were synthesized commercially. The primers sequences for nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), Kelch like-ECH-associated protein 1 (*Keap1*),  $\gamma$ -glutamylcysteine synthetase heavy (catalytic) subunit ( $\gamma$ -*Gcsh*), *Gstm3* and *Gapdh* were given in Table 1. A standard curve was prepared using a serial dilution of a reference sample, and was included in each real-time run to correct for possible variations in product amplification. Relative copy numbers were obtained from standard curve values, and were normalized to values obtained for the internal control, *Gapdh*. The fold change in expression was then obtained by  $2^{-\Delta\Delta CT}$  method.

#### 2.14. Western blot analysis for *Nrf2*, *Keap1*, $\gamma$ GCS, $\mu$ -GST and HO-1

The kidney tissues of control and experimental groups of rats were homogenized in 1 ml of ice-cold hypotonic buffer A containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl-fluoride (PMSF). To the tissue homogenate, 80  $\mu$ l of 10% Nonidet P-40 solution was added and the mixture was then centrifuged for 2 min at 14,000g. The supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed once with 500  $\mu$ l of buffer A plus 40  $\mu$ l of 10% Nonidet P-40, centrifuged, resuspended in 200  $\mu$ l of buffer C containing 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol and centrifuged for 5 min at 14,800g. The supernatant containing nuclear proteins was collected as a nuclear fraction for *Nrf2* analysis. Protein concentration was determined by the method of Lowry et al. (1951). Protein (10  $\mu$ g) was loaded into each well and subjected to immunoblot analysis for *Nrf2*, *Keap1*,  $\gamma$ -GCS,  $\mu$ -GST and heme oxygenase (HO)-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Band intensities were quantified by densitometric analysis and the results were normalized to the GAPDH expression in each group as percent of control.

### 2.16. *Histological observations of kidney*

A slice of kidney tissue was fixed in 10% formalin for 1 week at room temperature. Then the specimens were dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Tissue blocks were sectioned into 5  $\mu\text{m}$  thickness using a rotary microtome. Sections were stained by hematoxylin-eosin. Histological changes in the stained sections were viewed under the light microscope by a pathologist without prior knowledge of the groups.

### 2.17. *Transmission electron microscopic study*

A portion of kidney (about  $1\text{mm}^3$ ) from control and experimental groups of rats were fixed in 3% glutaraldehyde in 200 mM sodium phosphate buffer (pH 7.4) for 3 h at 4°C. Tissue samples were washed with the same buffer, post-fixed in 1% osmium tetroxide and 200 mM sodium phosphate buffer (pH 7.4) for 1 h at 4°C. The samples were again washed with the same buffer for 3 h at 4°C, dehydrated with graded series of ethanol and embedded in Araldite. Thin sections were cut with LKBUM4 ultramicrotome using a diamond knife (Diatome, Aldermaston, Berkshire, England), mounted on a copper grid and stained with 2% uranyl acetate and Reynolds lead citrate. The grids were examined under a Philips EM201C transmission electron microscope (Philips, Eindhoven, Netherlands).

### 2.18. *Statistical analysis*

The results were expressed as mean  $\pm$  SEM of six rats per group and statistical significance was evaluated by one-way ANOVA using SPSS (version 15.0) program followed by the post hoc test, least significant difference (LSD). Values were considered statistically significant when  $p < 0.05$ .

### 3. Results

#### 3.1. *Resveratrol improves insulin tolerance after insulin challenge in diabetic rats*

The effect of resveratrol as well as glyclazide treatment on the level of blood glucose in the control and experimental groups of rats receiving an intraperitoneal insulin challenge is shown in Figure 1. The blood glucose level is significantly ( $p < 0.05$ ) reduced in diabetic rats treated with resveratrol as well as glyclazide than that of diabetic group of rats. However, slight reduction of blood glucose is observed in diabetic group of rats in comparison with control group of rats. Conversely, there is no significant reduction in the blood glucose of control rats treated with resveratrol when compared with control group of rats. This assay indicated improved insulin sensitivity in resveratrol treated diabetic rats and is comparable with glyclazide treated diabetic group of rats.

#### 3.2. *Resveratrol normalizes renal dysfunction in diabetic rats*

Table 2 epitomizes the effect of resveratrol on the body weight, relative organ weight of kidney, creatinine clearance and the activities of renal AST, ALT and ALP in the control and experimental groups of rats. There was a significant increase ( $p < 0.05$ ) in the relative organ weight and the activities of renal AST, ALT and ALP in the diabetic group of rats with notable ( $p < 0.05$ ) decline in their body weight and creatinine clearance rate. However, control rats treated with resveratrol did not reveal any statistical significant on these indices when compared to that of control rats. Oral treatment with resveratrol as well as glyclazide to diabetic groups of rats significantly ( $p < 0.05$ ) normalized the altered levels when compared with diabetic group of rats.

#### 3.3. *Resveratrol enhances plasma adiponectin and C-peptide levels in diabetic rats*

Figure 2A,B depicts the effect of resveratrol on the levels of plasma adiponectin and plasma C-peptide in the control and experimental groups of rats. Oral treatment with resveratrol to control group of rats did not show any statistical significance on the levels of plasma adiponectin and C-peptide in comparison with control group of rats. Conversely, these levels were declined significantly ( $p<0.05$ ) in diabetic group of rats. However, resveratrol treatment, as in glyclazide treatment, to diabetic group of rats significantly ( $p<0.05$ ) improved these levels to near normalcy when compared with diabetic group of rats.

#### 3.4. *Resveratrol normalizes polyol pathway enzymes in diabetic kidney*

The activities of renal polyol pathway enzymes such as aldose reductase, sorbitol dehydrogenase and glyoxalase-I and level of AGEs formation are represented in Table 3. The diabetic group of rats demonstrated a significant ( $p<0.05$ ) escalation on the activities of renal aldose reductase and sorbitol dehydrogenase and level of AGEs with a significant ( $p<0.05$ ) decline in the activity of glyoxalase-I in comparison with control group of rats. Conversely, oral treatment with resveratrol as well as glyclazide to diabetic group of rats significantly ( $p<0.05$ ) normalized polyol pathway flux to near control values than that of diabetic group of rats. However, resveratrol treatment to control group of rats did not show any statistical difference in comparison to control group of rats.

#### 3.5. *Resveratrol abates proinflammatory cytokines in diabetic kidney*

The effect of oral treatment of resveratrol on the levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and NF- $\kappa$ B p65 subunit in renal tissues of control and experimental groups of rats are demonstrated in Figure 3A–D. The levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and NF- $\kappa$ B p65 subunit in control rats administered with resveratrol did not reveal any statistical difference when compared with that of control group of rats. Conversely, these proinflammatory cytokines



levels were escalated ( $p < 0.05$ ) significantly in diabetic group of rats in comparison with control group of rats. Moreover, oral treatment with resveratrol as well as glyclazide to diabetic group of rats significantly ( $p < 0.05$ ) altered the levels to near normalcy when compared with diabetic group of rats.

### 3.6. *Resveratrol scavenges superoxide, hydroxyl and NO radical formation in diabetic kidney*

Figure 4A–C illustrates the effect of resveratrol on the levels of superoxide anion, hydroxyl radical and NO in the renal tissues of control and experimental groups of rats. The levels of superoxide anion, hydroxyl radical and NO in diabetic group of rats were significantly ( $p < 0.05$ ) escalated when compared with control group of rats. Nevertheless, oral treatment of resveratrol as well as glyclazide to diabetic group of rats demonstrated a marked ( $p < 0.05$ ) decrement in the levels of superoxide anion, hydroxyl radical and NO. No considerable statistical variation was experienced in the control group of rats treated with resveratrol alone.

### 3.7. *Resveratrol restrains the formation of lipid peroxides, hydroperoxides and protein carbonyl in diabetic kidney*

The effect of resveratrol on the levels of lipid peroxides, hydroperoxides and protein carbonyls in the renal tissues of control and experimental groups of rats are illustrated in Table 4. No significant statistical deviation was calculated on the levels of renal lipid peroxides, hydroperoxides and protein carbonyls in control group of rats treated with resveratrol alone. On the other hand, there was a significant ( $p < 0.05$ ) escalation observed on the levels of lipid peroxides, hydroperoxides and protein carbonyls in renal tissues of diabetic

group of rats and were decreased ( $p<0.05$ ) significantly to near normalcy by the treatment of resveratrol as well as glyclazide to diabetic groups of rats.

### 3.8. *Resveratrol enhances the levels of non-enzymatic antioxidants in diabetic kidney*

The levels of renal non-enzymatic antioxidants such as vitamin C, vitamin E and GSH are exemplified in Table 5. The diabetic group of rats demonstrated a significant ( $p<0.05$ ) decline on the levels of renal non-enzymatic antioxidants in comparison with control group of rats. Conversely, oral treatment with resveratrol as well as glyclazide to diabetic group of rats significantly ( $p<0.05$ ) improved the levels to near control values than that of diabetic group of rats. However, resveratrol treatment to control group of rats did not show any statistical difference in comparison to control group of rats.

### 3.9. *Resveratrol fosters the activities of enzymatic antioxidants in diabetic kidney*

Table 6 indicates the effect of resveratrol on the activities of renal enzymatic antioxidants such as SOD, catalase, GPx, GST and GR in the control and experimental groups of rats. The activities were significantly ( $p<0.05$ ) diminished in the renal tissues of diabetic group of rats. Oral treatment of resveratrol, similar to that of glyclazide, significantly ( $p<0.05$ ) attenuated the altered activities of these enzymic antioxidants to near normalcy in renal tissues of diabetic rats. However, no significant statistical changes were observed in rats treated with resveratrol in comparison to that of control group of rats.

### 3.10. *Resveratrol normalizes the mRNA expression of Nrf2, Keap1, $\gamma$ Gcsh and Gstm3 in diabetic kidney*

The effect of resveratrol on the mRNA expression of *Nrf2*, *Keap1*,  *$\gamma$ Gcsh* and *Gstm3* in the kidney tissues of control and experimental groups of rats are depicted in Figure 5. The

mRNA expression of *Nrf2* and its downregulatory genes such as  $\gamma$ *Gcsh* and *Gstm3* were significantly ( $P < 0.05$ ) declined with simultaneous elevation of *Keap1* gene in the renal tissues of diabetic group of rats. However, these altered mRNA expressions were significantly ( $p < 0.05$ ) normalized in the diabetic group of rats by the oral administration of resveratrol as well as glyclazide. On the contrary, resveratrol treatment to control group of rats did not demonstrate any statistical difference in comparison to control group of rats.

### 3.11. *Resveratrol normalizes the protein expression of Nrf2, Keap1, $\gamma$ GCS, $\mu$ -GST and HO-1 in diabetic kidney*

Figure 5 depicts the effect of resveratrol on the protein expression of Nrf2, Keap1,  $\gamma$ -GCS,  $\mu$ -GST and HO-1 in the kidney tissues of control and experimental groups of rats. The expression of Nrf2 and its downregulatory proteins such as  $\gamma$ -GCS,  $\mu$ -GST and HO-1 were significantly ( $P < 0.05$ ) decreased with concomitant elevation in Keap1 in the renal tissues of diabetic group of rats. However, these altered protein expressions were significantly ( $p < 0.05$ ) normalized in the diabetic group of rats by the oral administration of resveratrol as well as glyclazide. Conversely, resveratrol treatment to control group of rats did not demonstrate any statistical difference in comparison to control group of rats.

### 3.12. *Resveratrol protects the histology of diabetic kidney*

Figure 6A–E depicts the photomicrographs of hematoxylin-eosin staining of renal tissues of control and experimental groups of rats. Figure 6A shows the section of renal tissue of control rats demonstrating normal architecture with normal glomeruli and tubules. Similarly, the sections of renal tissues of control group of rats treated with resveratrol also revealed an equivalent architecture (Fig. 6B). Figure 6C portrays the section of renal tissues of diabetic group of rats exhibiting tubular cell necrosis, tubular lumen dilation, foci of

denuded basement membrane, intraluminal casts, swelling or flattening of proximal tubular cells with brush border loss, diffuse interstitial edema, interstitial inflammatory cell infiltrates, vacuolization, pyknotic nuclei, medullary congestion, hyaline casts, epical blebbing and decreased cellularity of the glomeruli. Figure 6D demonstrates the section of renal tissues of diabetic group of rats treated with resveratrol presenting a normal glomerular, renal tubule and interstitial tissue appearance. Likewise, the renal tissues of diabetic rats treated with glyclazide shows similar pattern of renal histological architecture (Fig. 6E) and are comparable with control group of rats.

### 3.13. *Resveratrol protects the ultrastructure of diabetic kidney*

The ultrastructural changes occurred in kidney of control and experimental groups of rats are shown in Figure 7A–E. Figure 7A represents the electron micrograph of kidney of control group of rats showing the normal glomeruli, renal tubules, podocytes and mesangial cells. Similar architecture is observed in the electron micrograph of control group of rats treated with resveratrol (Fig. 7B). The electron micrograph of kidney of diabetic group of rats (Fig. 7C) revealed the glomerular filtration barrier i.e. thickened basement membrane, decreased number of podocytes, loss of podocytes foot processes, reduction of mesangial cells around the filtration barrier, capillary with fenestrated endothelial layer, disruption of basal infoldings of proximal and distal convoluted tubules, decreased number of mitochondria and fibrosis of proximal convoluted tubules. The electron micrograph (Fig. 7D) apparently shows the renal tissue protective nature of resveratrol in diabetic group of rats by virtue of an apparent appearance of normal architecture. Likewise, the electron micrograph of kidney of diabetic group of rats treated with glyclazide showing similar pattern of renal tissue protection (Fig. 7E) and are comparable with control group of rats.

## 4. Discussion

The present investigation demonstrates the renoprotective potential of resveratrol against hyperglycemia-mediated oxidative stress in streptozotocin-nicotinamide-induced diabetic nephropathy. Diabetic nephropathy is one of the major microvascular complications and is a leading cause of end-stage renal disease. The pathogenesis of diabetic nephropathy is multifactorial in which chronic hyperglycemia plays a crucial role [43]. During diabetic milieu, supraphysiological glucose is involved in the formation of AGEs and the mitochondrial production of free radicals and consequently, to cell death and renal dysfunction. Thus, oxidative stress plays a decisive role in the development of diabetic nephropathy, which is characterized by thickening of glomerular basement membranes, expansion of mesangial cells, glomerular hypertrophy and loss of podocytes, expansion of tubular basement membranes, tubular atrophy, interstitial fibrosis and arteriosclerosis [44].

The pathophysiological alterations and deteriorated renal functions established by chronic hyperglycemia-mediated oxidative stress in the experimental rats are closely similar to that of human diabetic nephropathy. Further, streptozotocin-nicotinamide-induced experimental diabetes is exemplified by moderate and stable hyperglycemia, glucose intolerance and significantly altered glucose-stimulated insulin secretion that contributes number of features comparable to human diabetes [21]. The elevated blood glucose, increased kidney weight, altered intraperitoneal insulin tolerance, increased activities of renal AST, ALT and ALP and diminished creatinine clearance in the diabetic group of rats indicate the development of renal hypertrophy, glomerular injury and renal dysfunction. However, oral treatment with resveratrol to diabetic group of rats normalized these altered levels into near normalcy suggesting that resveratrol effectively ameliorate diabetic renal injury in addition with attenuating oxidative stress as well as limiting the activation of polyol pathway.

Adiponectin, an adipose-specific protein, is an insulin-sensitizing, antiinflammatory and vasculo-protective cytokine and has been shown to improve glucose tolerance and insulin resistance in experimental animals [45] and humans [46]. The elevated level of plasma adiponectin is directly interrelated with reduced likelihood of moderate diabetic renal dysfunction [47]. The increased levels of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 in experimental diabetes may be responsible for the decline in level of plasma adiponectin [48]. The results of the present investigation revealed that oral treatment with resveratrol may improve the renal function by escalating the plasma adiponectin concentration in the diabetic rats. However, the mechanisms by which resveratrol treatment fosters the level of circulating adiponectin is unknown, but it is possible that inhibition of TNF- $\alpha$  increases the secretion of both plasminogen activator inhibitor-1 and IL-6 and recovers the insulin resistance, resulting in an elevation of adiponectin [49]. Thus, resveratrol might enhance plasma adiponectin level through its anti-inflammatory mechanisms.

C-peptide is a cleavage product of insulin biosynthesis and is subsequently released along with insulin in equimolar quantities to the circulation. It is considered to be a reliable marker of residual  $\beta$ -cell function. The prime task of C-peptide is to facilitate proper folding of proinsulin by assisting disulfide bonds formation within A- and B-chains [50]. It possesses insulin-mimetic property thereby activates insulin receptors and increases the rate of glycogenesis and diminishes glucose-stimulated insulin secretion in rats. C-peptide is reported to alleviate incipient diabetic nephropathy by reducing glomerular hyperfiltration, improving functional reserve, reducing urinary albumin excretion and preventing glomerular and renal hypertrophy in diabetic rats [51]. The oral administration of resveratrol to diabetic group of rats elevated the C-peptide level through activating remnant pancreatic  $\beta$ -cells.

It is well-known that diabetes is associated with chronic hyperglycemia and one of the foremost consequences of hyperglycemia is an increased rate of oxidative phosphorylation as well as the activation of the polyol pathway. Aldose reductase, the first and rate-limiting enzyme in polyol pathway, catalyzes the NADPH dependent reduction of glucose to sorbitol. Sorbitol is consequently converted to fructose by sorbitol dehydrogenase with  $\text{NAD}^+$  as cofactor. The fructose and its metabolites are involved in the non-enzymatic glycation of cellular proteins and lipids in the kidney and other organs, which aggravate AGEs formation and glycative injury. Intracellular accumulation of sorbitol impedes myo-inositol uptake and its metabolism and declines  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  activity [52, 53], leading to dysfunction in renal tubular reabsorption. The oxidation of sorbitol by  $\text{NAD}^+$  elevates cytosolic  $\text{NADH}:\text{NAD}^+$  ratio, thereby hindering the glyceraldehyde-3-phosphate dehydrogenase activity and increasing triose phosphate level. Enhanced triose phosphate concentrations could augment the formation of both methylglyoxal, a precursor of AGEs, and diacylglycerol, thus activating protein kinase C signaling [54]. Hence, the augmented polyol pathway flux has been suggested to induce overexpression of various cytokines and signaling proteins that are responsible for the thickening of the glomerular basement membrane and progressive accumulation of extracellular matrix components in the mesangium. Thus, both oxidative stress and increased aldose reductase and sorbitol dehydrogenase activities are implicated in the pathogenesis of diabetic nephropathy [55].

Glyoxalase-I is a GSH-dependent detoxifying enzyme involved in metabolism of methylglyoxal thereby suppressed the available precursors for AGEs formation under hyperglycemic conditions. Because the elevated production of methylglyoxal, a precursor for AGEs formation, has been directly associated to the development of diabetic nephropathy [56]. Thus glyoxalase-I is involved in the metabolism of these physiological reactive carbonyl compounds in tissues especially in the kidney, thereby glycative stress could be

attenuated. Various studies have signified that elevated activity or expression of glyoxalase-I could decline AGEs formation via lowering their precursors such as methylglyoxal [57, 58]. The results of the present study indicated that oral administration of resveratrol improved the glyoxalase-I activity, which further efficiently metabolized methylglyoxal. Thus, these enzymes have been considered as therapeutic targets for improving glycation associated diabetic complications including diabetic nephropathy. Moreover, the present investigation reveal that oral administration of resveratrol moderated renal activity of aldose reductase and sorbitol dehydrogenase and improved the activity of glyoxalase-I, which consequently led to declined formation of sorbitol and fructose in kidney. This observation indicated that resveratrol could assuage diabetic renal glycative damage via restraining polyol pathway and declining AGEs formation.

Inflammation and pro-inflammatory cytokines have been suggested to play a significant role in the development and progression of diabetic nephropathy. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are essentially produced by intrinsic renal cells together with dendritic, endothelial, glomerular, mesangial and renal tubular cells, because the expression and synthesis of these cytokines is not only restricted to hematopoietic cells. Therefore, these cytokines, acting in a paracrine or autocrine manner, may induce a variety of effects on different renal structures. The significant elevation in the wet weight of kidney is an accurate index of renal hypertrophy and one of the earliest renal changes during diabetes and directly related to renal TNF- $\alpha$ , IL-1 $\beta$  and IL-6 expressions [59].

TNF- $\alpha$  is cytotoxic to glomerular, mesangial and epithelial cells and competent to induce direct renal injury through the generation of reactive free radicals in various cells, including mesangial cells. IL-1 $\beta$  is also involved in the development of abnormalities in intraglomerular hemodynamics related to prostaglandin synthesis by mesangial cells [60]. IL-



$1\beta$  together with  $\text{TNF-}\alpha$  may provoke the expression of iNOS in glomerular mesangial cells, which results in the production of large amounts of NO and causes an elevation of cellular cGMP concentrations. Moreover, streptozotocin-nicotinamide-induced diabetic nephropathy is also associated with nitrosative stress; streptozotocin itself is a NO donor and liberates this reactive species during its intracellular metabolism. The excessive formation of NO and cGMP by mesangial cells not only alters the contractile responses of the cells but also may cause tissue injury and thus contribute to hyperfiltration and microalbuminuria that characterizes early diabetic nephropathy [61, 62].

IL-6 accelerates mesangial cell proliferation, enhances fibronectin expression, affects extracellular matrix dynamics at both mesangial and podocyte levels and increases endothelial permeability. Recent investigations on diabetics established a significant association between IL-6 and glomerular basement membrane thickening, a crucial lesion of diabetic nephropathy and a strong predictor of renal progression [63]. During hyperglycemia, increased activation of NF- $\kappa$ B is closely associated with regulation of mesangial cell proliferation that has been suggested to be a key step in the pathogenesis of diabetic nephropathy. Then the activated NF- $\kappa$ B is translocated into the nucleus and results in the transcriptional expression of genes associated with cellular growth properties. This may contribute to the accumulation of renal extracellular matrix and tubulointerstitial fibrosis, resulting in kidney damage [64]. The present study indicate that the oral administration of resveratrol to diabetic rats significantly attenuates the elevated levels of renal  $\text{TNF-}\alpha$ , IL- $1\beta$  and IL-6 in experimental diabetic rats to near normalcy, thereby moderates the consequence of inflammation-mediated oxidative tissue damage.

There is coercing evidence that surplus level of superoxide generated by diabetic hyperglycemia plays a vital role in diabetic kidney damage. The total amassing of superoxide

anion is determined by a balance between superoxide anion production and antioxidant competence. Elevated glucose flux enhances the generation of superoxide anion by mitochondrial electron-transport chain and the overproduced superoxide anion augments the major pathways such as protein kinase C, AGE products and hexosamine pathways [65]. Various pathogenic pathways of diabetes such as increased NADPH oxidase activity, uncoupled endothelial nitric oxide synthase and enhanced signaling of AGEs, angiotensin II and oxidized-LDL receptors is also responsible for inexorable generation of superoxide anion. Further, the elevated generation of superoxide anion leads to the formation of secondary reactive free radicals including peroxynitrite and hydroxyl radicals, leading the damage of DNA, proteins and lipids and triggers renal cell injury [66]. The instantaneous overproduction of NO and superoxide facilitates the production of a cytotoxic peroxynitrite anion that in turn oxidizes sulfhydryl groups in proteins, instigates lipid peroxidation and nitrates amino acids such as tyrosine, which affects several signal transduction pathways [67]. Thus free radicals are implicated in the cell destruction and progression of kidney tubular damage and the results of the present investigation speculate that these free radicals could be significant in the pathogenesis of renal tissue damage at the later stages of experimental diabetes mellitus. However, oral treatment of resveratrol efficiently scavenges these free radicals thereby protects the kidney tissue from free radicals-mediated damage.

Lipid peroxides, hydroperoxides and protein carbonyls are the secondary products of oxidative stress and are unleashed as a result of the toxic effect of reactive oxygen species produced during lipid peroxidation in diabetes. Lipid peroxidation may also play a crucial role in diabetic glomerulosclerosis. The initial hyperfiltering phase of diabetic kidney disease, heralded by the presence of microalbuminuria, is followed by gross proteinuria, progressive diminution in glomerular filtration rate and excessive amassing of extracellular matrix proteins in the glomerular capillaries and mesangium. The latter process leads the way to

eventual glomerulosclerosis and renal failure [68]. The results of the present study also inline with the previous studies, in which resveratrol administration to diabetic group of rats notably declined the levels of renal lipid peroxides, hydroperoxides and protein carbonyls [69]. This normalization may be accomplished by the antioxidant and free-radical quenching nature of resveratrol, because resveratrol is reported to protect the cells from peroxidative stress and lipid peroxidation as well as oxidized lipoproteins-mediated cell death [70, 71].

Chronic hyperglycemia often associated with a notable decline in the level of intracellular antioxidants and elevation in the formation of pro-oxidants such as reactive free radicals and electrophilic substances that eventually results in renal dysfunction and deterioration. There are a number of enzymatic and nonenzymatic sources of reactive free radicals in the diabetic kidney, such as autoxidation of glucose, transition metal-catalyzed Fenton reactions, AGEs, polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase and NAD(P)H oxidase [44]. Such glucotoxicity possibly related to the vulnerability of renal tissues to oxidative stress, because both the functional activities and expression levels of antioxidant enzymes such as SOD, catalase, GPx, GST and GR, are forced to overcome the susceptibility rates. SOD, a chief antioxidant enzyme for the removal of superoxide anion, dismutates superoxide into hydrogen peroxide and molecular oxygen. The hydrogen peroxide is further detoxified to water by catalase or GPx. In addition, GPx is involved in diminution of highly cytotoxic products such as lipid peroxides and other organic hydroperoxides. GSH is an essential cosubstrate for the activity of GPx that oxidizes the GSH into oxidized glutathione. Oxidized glutathione is recycled back to glutathione by GR, through an NADPH consuming process [72]. Hence, the activities of these key enzymatic antioxidants are diminished during oxidative stress. However, oral treatment of resveratrol significantly improved the activities of enzymatic antioxidants. In this context, various studies have reported the diminished

activities of these antioxidant enzymes in the diabetic kidney and several compounds with antioxidant activity have been shown to normalize activities of these antioxidant enzymes in diabetic rats [18, 73, 74].

The inexorable generation of reactive free radicals and lipid peroxides during diabetes-mediated oxidative stress could be correlated to decline in the level of non-enzymatic antioxidants such as GSH, vitamin C and vitamin E. GSH improves the scavenging capacity against free radicals induced oxidative stress in diabetic rats. Polyphenols are reported to enhance the activity of  $\gamma$ -glutamylcysteine synthetase and demonstrated simultaneous escalation in the intracellular glutathione level [75]. In addition to GSH, vitamin C and vitamin E are interrelated by recycling processes. Because, recycling of tocopheroxyl radicals to tocopherol is accomplished by reaction with ascorbic acid and dehydroascorbic acid is also produced in reaction with GSH. Studies have revealed the existence of a liable glutathione dependent factor, which recycles the tocopheroxyl radicals to tocopherol. If recycling of tocopheroxyl radicals to tocopherol is a key mechanism for preservation of tissue tocopherol level, ascorbic acid insufficiency is predictable to result in exhaustion of tissue tocopherol. In the present study, the levels of GSH, vitamin C and vitamin E are declined in the diabetic kidney portending the recycling of tocopheroxyl radicals to tocopherol may have been impeded that results in elevated lipid peroxidation [76]. However, the oral administration of resveratrol to diabetic rats notably elevated the renal levels of GSH, vitamin C and vitamin E. It is evidenced that polyphenols like resveratrol, owing to their intermediate redox potential and physiochemical characteristics, can probably act an interface between vitamin C and vitamin E [77].

Nrf2, a member of the cap-N-collar family, is the key transcription factor that regulates antioxidant response element-mediated expression of detoxifying antioxidant

enzymes [78]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by an actin binding repressor protein-Keap1. Upon exposure of cells to oxidative stress or electrophilic compounds with the capacity to oxidize or covalently transform thiol groups, Nrf2 easily dissociates from its repressor protein, translocates into the nucleus, binds to antioxidant response elements and transactivates the genes of detoxifying and antioxidant enzymes such as  $\gamma$ -GCL,  $\mu$ -GST and HO-1 [79]. During hyperglycemia-mediated oxidative stress, the expression of Nrf2 and its downregulatory enzymes like  $\gamma$ -GCL,  $\mu$ -GST and HO-1 were significantly decreased in the renal tissues of diabetic rats. However, resveratrol treatment significantly modulates the expression of Nrf2 in hyperglycemia-mediated oxidative stress by upregulation of  $\gamma$ -GCL,  $\mu$ -GST and HO-1.

The renal tissue defensive nature of resveratrol in control and experimental groups of rats were ascertained by histological as well as ultrastructural studies, respectively. The diabetogenic potential of streptozotocin is accountable for a progressive development of the renal tissues lesions. The major morphological abnormalities observed in the diabetic kidney was tubular cell swelling, cellular vacuolization, pyknotic nuclei, medullary congestion, severe lesions and necrosis in a large number of proximal tubular cells, hemorrhagic and hyaline cast deposits, apical blebbing, glomerulus hypertrophy, expanded and congested glomerular capillaries, lysis in tubular epithelial cells and irregular tubular lumens, with reference to control rats [80]. Transmission electron microscopic studies also potentiate the histological observations showing the dysfunction and deterioration of the diabetic kidney ultrastructure. The electron microscopy of diabetic kidney has revealed disruption of the podocytes foot processes, with disruption of the basal infoldings and loss of mitochondria between the folds of proximal convoluted tubules. Mesangiolysis, subendothelial edema as well as focal podocyte degeneration and detachment were also observed along with enlargement of the inner layer of glomerular basement membrane and disruption of fibrils in

the middle layer of glomerular basement membrane and in mesangium of the kidney tissues of diabetic rats [81, 82]. However, administration of resveratrol to diabetic rats ameliorated all of those ultrastructural alterations in the kidney tissues confirming its protective nature during diabetes. There are no such studies available on the protective nature of resveratrol on the kidney tissues ultrastructure during experimental diabetes. Hence, this investigation should be considered an innovative assessment for the renal tissue protective nature of resveratrol in rats insulted by streptozotocin.

Thus, the present study divulges the renoprotective nature of resveratrol in streptozotocin-nicotinamide-induced diabetic rats and provides evidence that the protective effects are, possibly, due to the decline in oxidants and proinflammatory cytokines production by the renal tissues. Moreover, resveratrol treatment to streptozotocin-nicotinamide-induced diabetic rats exhibited a significant ameliorative potential probably by attenuating the hyperglycemia-mediated oxidative stress thereby alleviating the ultrastructural alterations such as tubulonecrosis, glomerulosclerosis, glomerular basement membrane thickening and changes in the proximal and convoluted tubules. Further detailed studies are in progress to explicate the precise mechanism by which resveratrol reduces its renoprotective potentials.

### **Acknowledgements**

The financial assistance from University Grants Commission (UGC), New Delhi, India is gratefully acknowledged. The authors wish to record their sincere thanks to Ms.B.Rita and Mr.P.Srinivasan, The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College and Hospital, Vellore-632004, India, for their help in transmission electron microscopic and histopathological studies.

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**Figure Legends**

**Fig. 1.** Effect of resveratrol on the intraperitoneal insulin tolerance test in control and experimental groups of rats. Values are expressed as mean  $\pm$  SEM for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test LSD. Asterisk (\*) indicating a  $p < 0.05$  versus control rats; Double asterisk (\*\*) indicating a  $p < 0.05$  versus diabetic rats.

**Fig. 2.** Effect of resveratrol on the plasma levels of (A) adiponectin and (B) C-peptide in the control and experimental groups of rats. Values are expressed as mean  $\pm$  SEM for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test LSD. Asterisk (\*) indicating a  $p < 0.05$  versus control rats; Double asterisk (\*\*) indicating a  $p < 0.05$  versus diabetic rats.

**Fig. 3.** Effect of resveratrol on the renal levels of (A) TNF- $\alpha$ , (B) IL-1 $\beta$ , (C) IL-6 and (D) NF- $\kappa$ B p65 subunit in the control and experimental groups of rats. Values are expressed as mean  $\pm$  SEM for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test LSD. Asterisk (\*) indicating a  $p < 0.05$  versus control rats; Double asterisk (\*\*) indicating a  $p < 0.05$  versus diabetic rats.

**Fig. 4.** Effect of resveratrol on the renal levels of (A) superoxide anion, (B) hydroxyl radical and (C) NO in control and experimental groups of rats. Values are expressed as mean  $\pm$  SEM for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test LSD. Asterisk (\*) indicating a  $p < 0.05$  versus control rats; Double asterisk (\*\*) indicating a  $p < 0.05$  versus diabetic rats.

**Fig. 5.** Effect of resveratrol on the renal mRNA expression levels of *Nrf2*, *Keap1*,  *$\gamma$ -Gcsh* and *Gstm3* in control and experimental groups of rats. Values are expressed as mean  $\pm$  SEM for groups of six rats in each. Statistical significance was determined by one way ANOVA

followed by post hoc test LSD. Asterisk (\*) indicating a  $p < 0.05$  versus control rats; Double asterisk (\*\*) indicating a  $p < 0.05$  versus diabetic rats.

**Fig. 6.** Effect of resveratrol on the renal protein expression levels of Nrf2, Keap1,  $\gamma$ -GCS,  $\mu$ -GST and HO-1 in control and experimental groups of rats. Values are expressed as mean  $\pm$  SEM for groups of six rats in each. Statistical significance was determined by one way ANOVA followed by post hoc test LSD. Asterisk (\*) indicating a  $p < 0.05$  versus control rats; Double asterisk (\*\*) indicating a  $p < 0.05$  versus diabetic rats.

**Fig. 7.** Photomicrographs of hematoxylin-eosin staining of renal tissues of control and experimental groups of rats. Diabetes was induced by single intraperitoneal injection of streptozotocin (50 mg/kg body weight), 15 min after the intraperitoneal administration of nicotinamide (110 mg/kg body weight). Resveratrol (5 mg/kg body weight) was orally administered daily for 30 days, as described under Materials and Methods Section. At the end of experimental period, the rats were killed and the renal tissues were sectioned for the histological studies. Histological photograph of (A) Control, (B) Control + Resveratrol, (C) Diabetic, (D) Diabetic + Resveratrol and (E) Diabetic + Glyclazide treated rats' renal tissue sections were showed at 100x magnification. Distal tubules (DT), Glomerulus (G) and Proximal tubules (PT). Scale bar 0.1 mm.

**Fig. 8.** Transmission electron micrographs of renal tissues of control and experimental groups of rats. Diabetes was induced by single intraperitoneal injection of streptozotocin (50 mg/kg body weight), 15 min after the intraperitoneal administration of nicotinamide (110 mg/kg body weight). Resveratrol (5 mg/kg body weight) was orally administered daily for 30 days, as described under Materials and Methods Section. At the end of experimental period, the rats were sacrificed and the renal tissues were sectioned for the transmission electron microscopic studies. Transmission electron micrographs of (A) Control, (B) Control +

Resveratrol, (C) Diabetic, (D) Diabetic + Resveratrol and (E) Diabetic + Glyclazide treated rats' renal tissue sections were showed at x2000, x1500, x15000, x10000, x4500 magnification, respectively. Basement membrane (BM), Capillary loop (CL), Endothelial fenestrations (EF), Mesangial cell (MC), Microtubules (MT), Nucleus (N), Podocytes (P), Red blood cells (R), Urinary space (US). Scale bar 1  $\mu\text{m}$ .

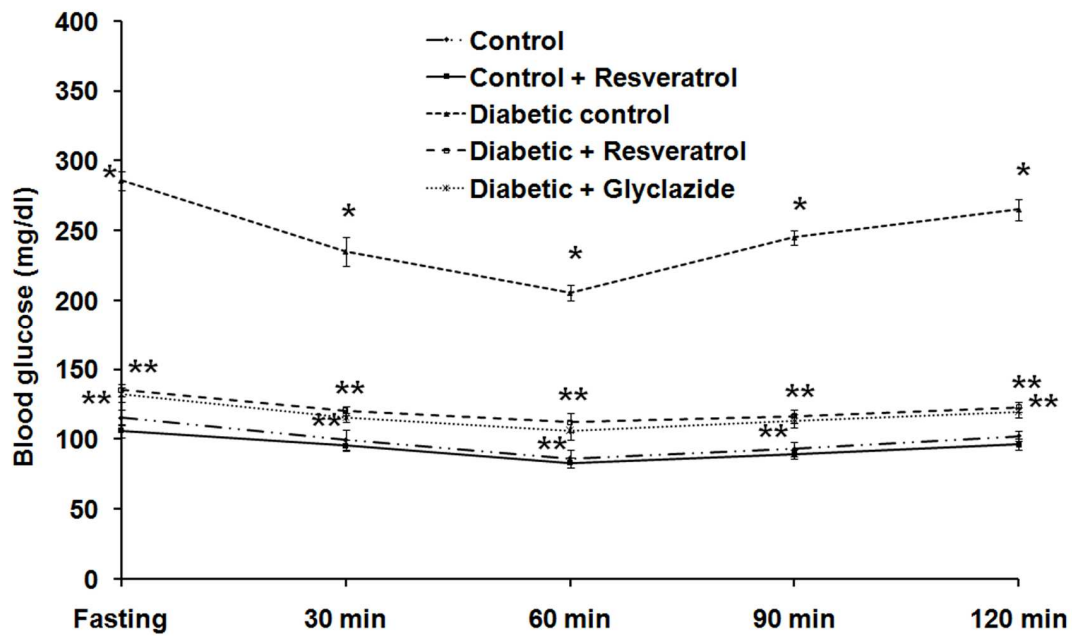


Fig. 1

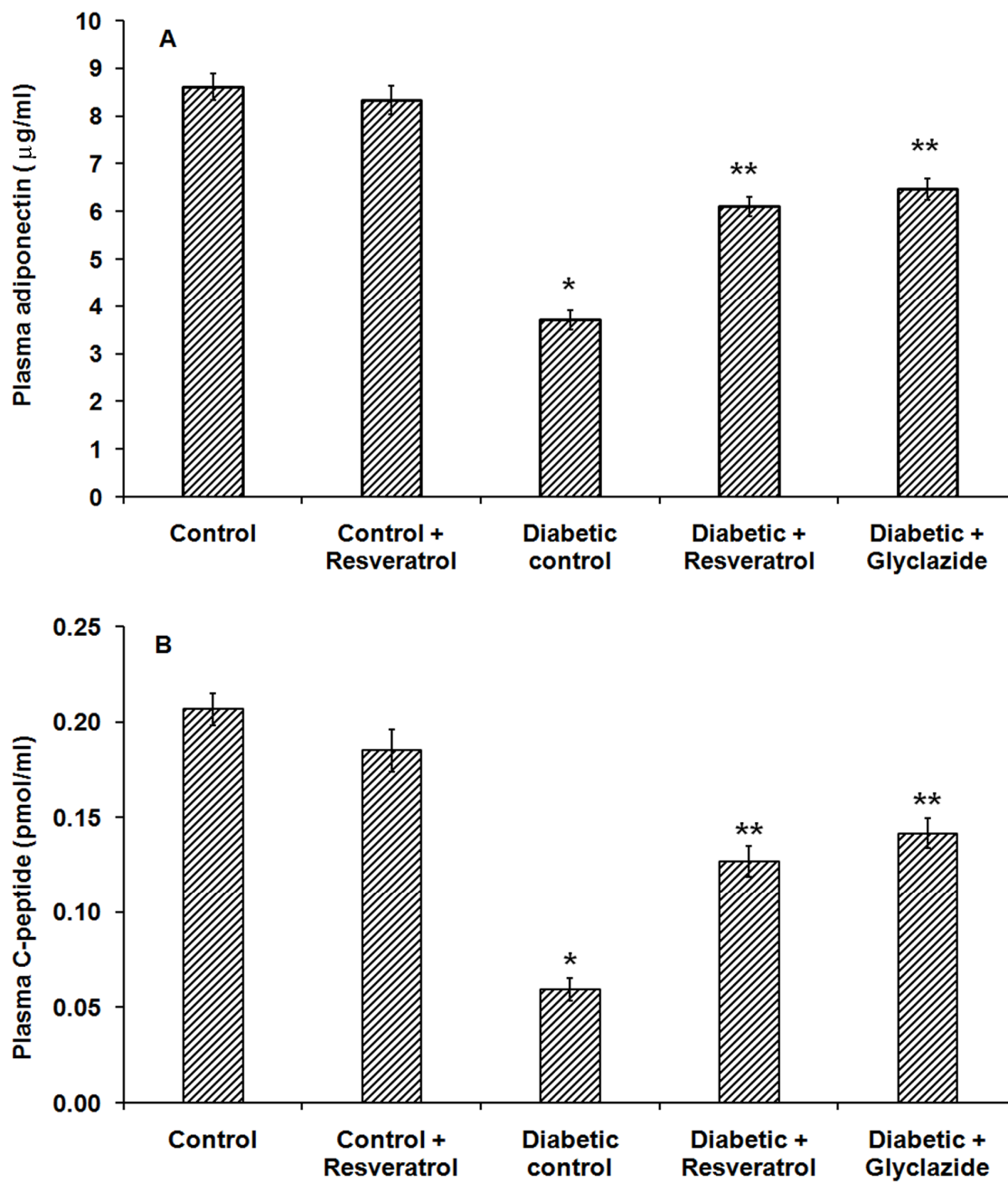


Fig. 2



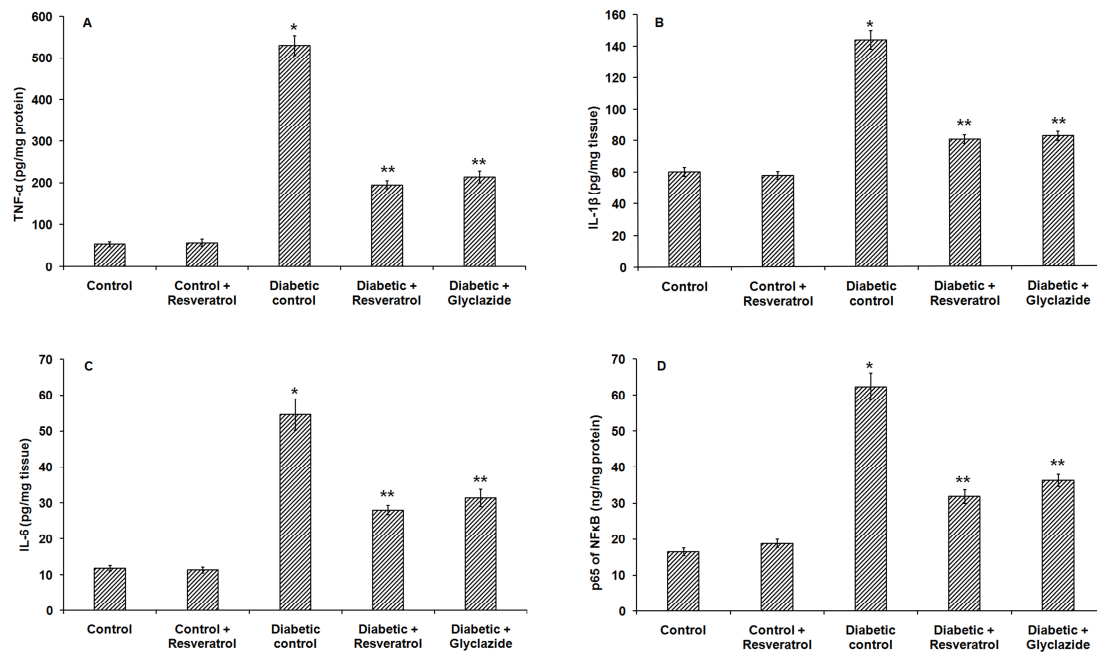


Fig. 3

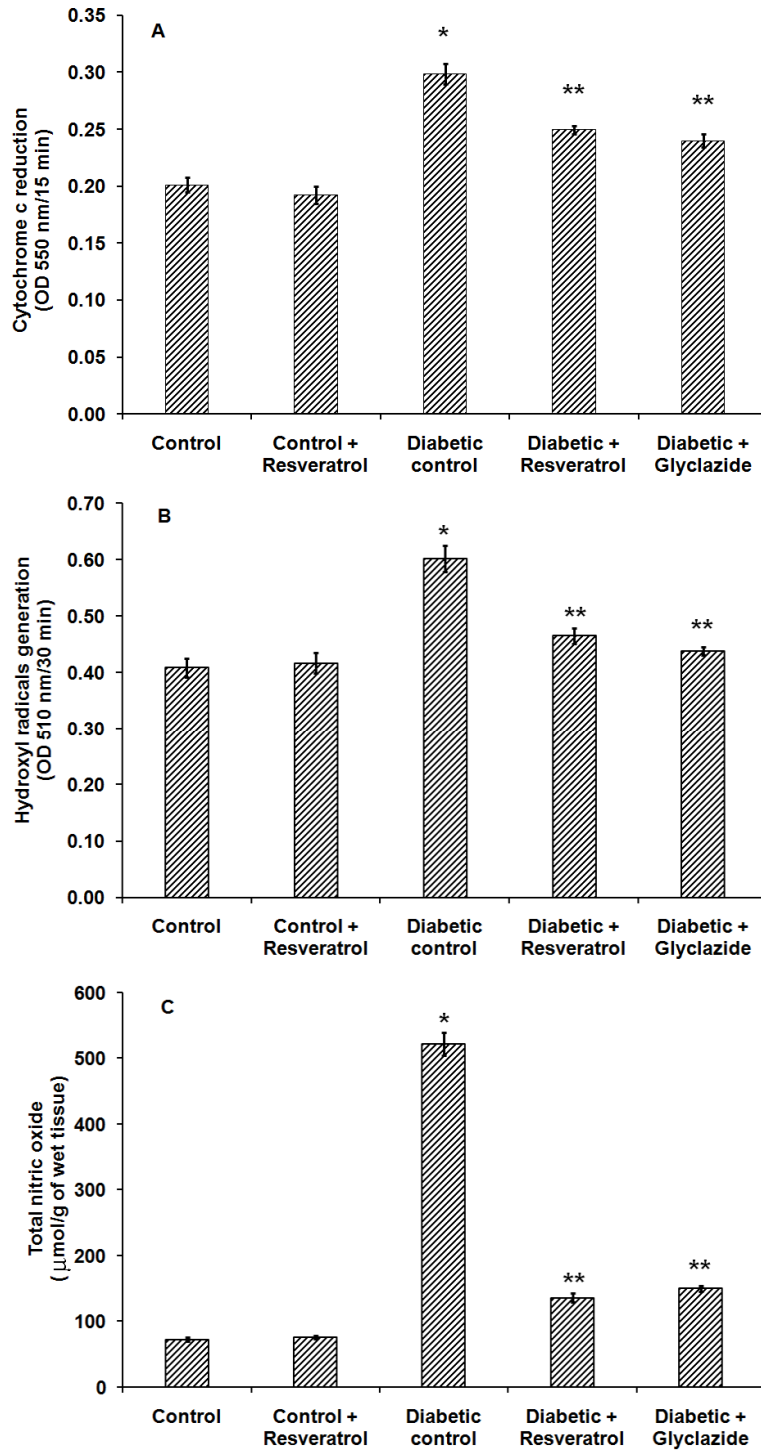


Fig. 4

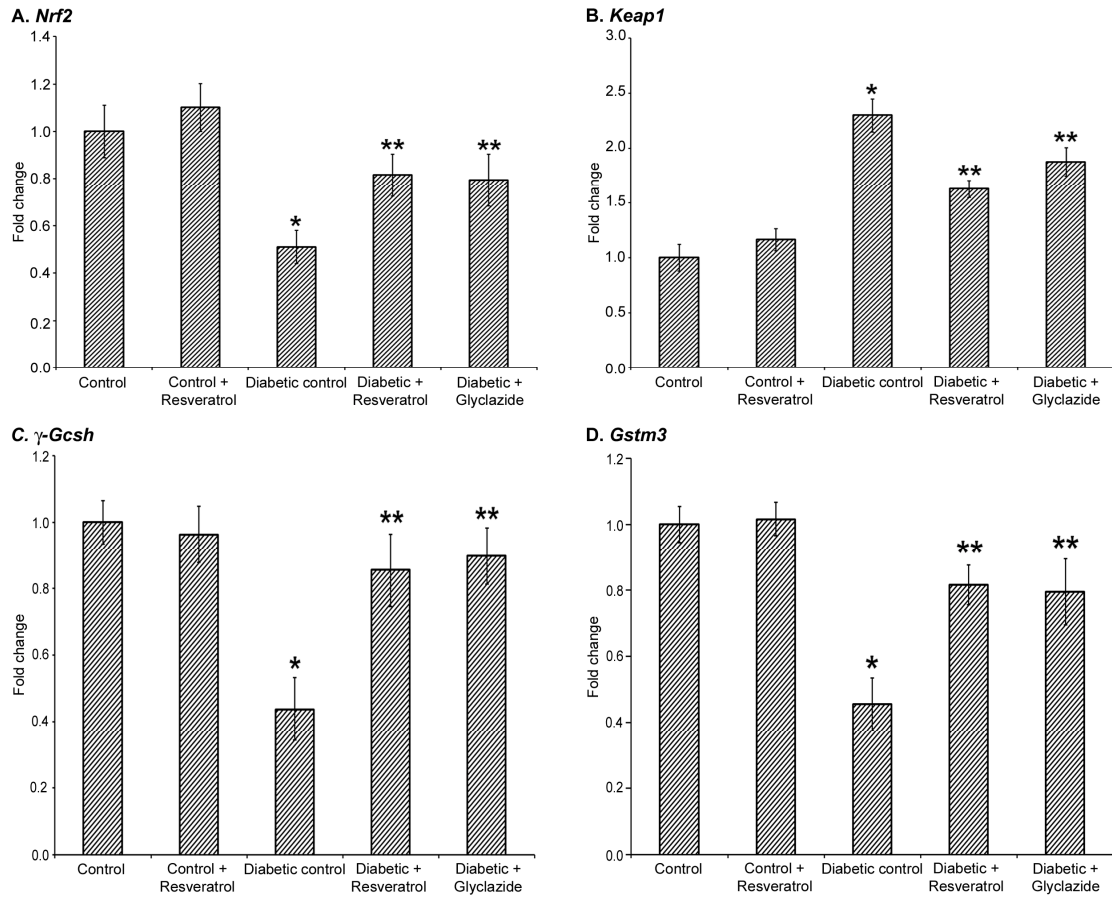


Fig. 5

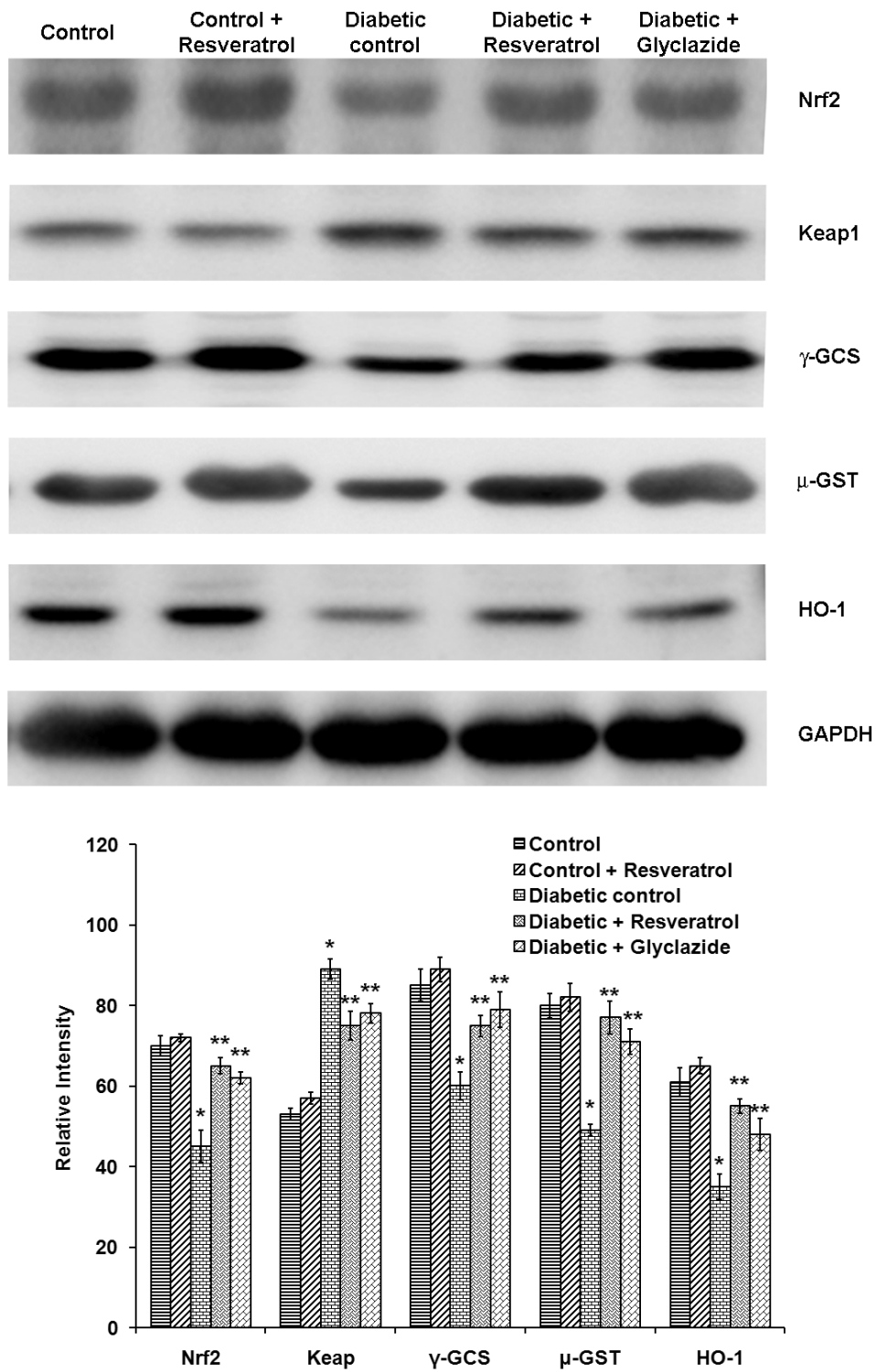


Fig. 6

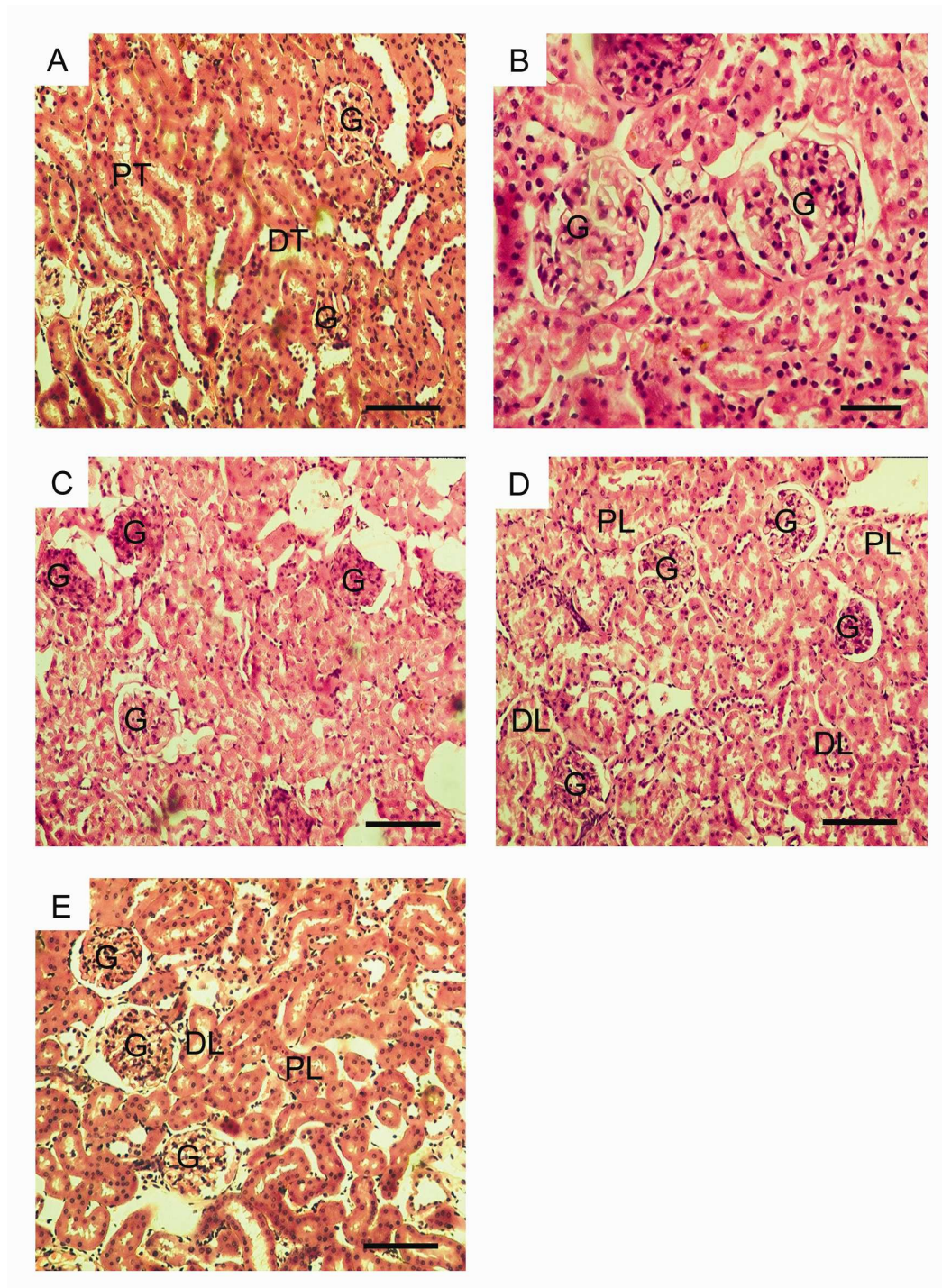


Fig. 7



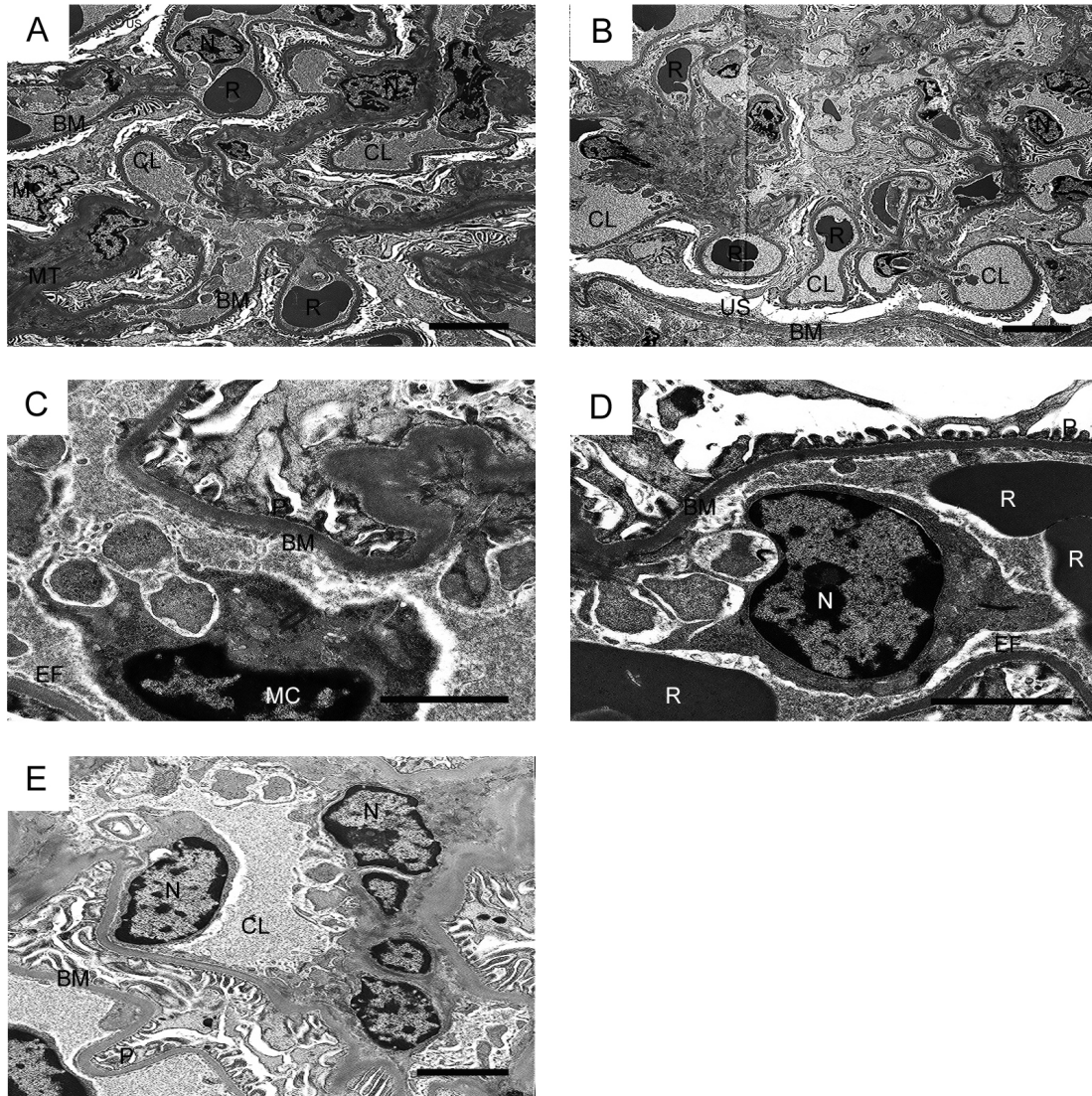


Fig. 8

TABLE 1. List of primer sequences for real-time qPCR

Accession No.	Gene Description	Gene symbol	Forward	Reverse	Product size
NM_031789.1	Nuclear factor (erythroid-derived 2)-like 2	<i>Nrf2</i>	gagacggccatga ctgat	gtgaggggatcgat gagtaa	196
NM_057152.1	Kelch-like ECH-associated protein 1	<i>Keap1</i>	ctgcatccaccaca gcagcgt	gtgcagcacacaga ccccggc	250
NM_012815.2	$\gamma$ -Glutamylcysteine synthetase heavy subunit	$\gamma$ - <i>Gcsh</i>	atgcagtattctgaa ctacc	acaaactcagattca cctac	396
NM_020540.1	Glutathione-S-transferase, mu 3	<i>Gstm3</i>	gaacgttcgaggac ttactca	acgtatctcttctcctc atagttg	78
NM_017008.3	Glyceraldehyde-3-phosphate dehydrogenase	<i>Gapdh</i>	aggttgtctcctgtg acttc	ctggtgctgtagccat attc	130

TABLE 2. Effect of resveratrol on the body weight, relative kidney weight, creatinine clearance and the activities of renal AST, ALT and ALP in the control and experimental groups of rats

Groups	Body weight (g)	Relative kidney weight	Creatinine clearance	AST	ALT	ALP
Control	180.15 ± 2.18	0.95 ± 0.02	2.66 ± 0.33	54.09 ± 2.01	150.78 ± 9.64	0.23 ± 0.02
Control + Resveratrol	183.29 ± 3.14	0.97 ± 0.01	2.67 ± 0.52	52.65 ± 2.38	147.11 ± 4.93	0.22 ± 0.01
Diabetic control	138.68 ± 2.34 <sup>a</sup>	1.52 ± 0.05 <sup>a</sup>	1.35 ± 0.05 <sup>a</sup>	107.33 ± 5.74 <sup>a</sup>	293.68 ± 9.22 <sup>a</sup>	0.56 ± 0.05 <sup>a</sup>
Diabetic + Resveratrol	165.09 ± 3.08 <sup>b</sup>	1.09 ± 0.02 <sup>b</sup>	2.16 ± 0.17 <sup>b</sup>	69.80 ± 1.98 <sup>b</sup>	176.67 ± 5.51 <sup>b</sup>	0.42 ± 0.02 <sup>b</sup>
Diabetic + Glyclazide	169.11 ± 4.03 <sup>b</sup>	1.12 ± 0.02 <sup>b</sup>	2.09 ± 0.28 <sup>b</sup>	75.11 ± 2.61 <sup>b</sup>	183.35 ± 6.13 <sup>b</sup>	0.45 ± 0.01 <sup>b</sup>

Values are given as mean ± SEM for groups of six rats in each. One way ANOVA followed by post hoc test LSD. Values are statistically significant at  $p < 0.05$ . <sup>a</sup> Diabetic rats were compared with control rats; <sup>b</sup> Diabetic + Resveratrol and Diabetic + Glyclazide treated diabetic rats were compared with diabetic rats.

Units: Creatinine clearance – mL/min/100 g body weight; AST and ALT – nmoles of pyruvate/h/mg protein; ALP - μmoles of phenol liberated/min/mg protein.



TABLE 3. Effect of resveratrol on the renal activities of aldose reductase, sorbitol dehydrogenase and glyoxalase-I and serum level of AGEs in control and experimental groups of rats

Groups		Aldose reductase	Sorbitol dehydrogenase	Glyoxalase-I	Serum AGEs
Control		1.13 ± 0.05	4.62 ± 0.16	288.60 ± 7.14	488.50 ± 23.96
Control + Resveratrol	+	1.19 ± 0.08	4.55 ± 0.15	302.10 ± 5.87	509.20 ± 17.34
Diabetic control		2.76 ± 0.12 <sup>a</sup>	9.54 ± 0.57 <sup>a</sup>	132.50 ± 6.86 <sup>a</sup>	945.50 ± 19.29 <sup>a</sup>
Diabetic + Resveratrol	+	1.69 ± 0.07 <sup>b</sup>	6.56 ± 0.36 <sup>b</sup>	205.30 ± 6.79 <sup>b</sup>	749.80 ± 15.34 <sup>b</sup>
Diabetic + Glyclazide	+	1.75 ± 0.08 <sup>b</sup>	7.36 ± 0.33 <sup>b</sup>	213.85 ± 5.99 <sup>b</sup>	722.60 ± 12.07 <sup>b</sup>

Values are given as mean ± SEM for groups of six rats in each. One way ANOVA followed by post hoc test LSD. Values are statistically significant at  $p < 0.05$ . <sup>a</sup> Diabetic rats were compared with control rats; <sup>b</sup> Diabetic + Resveratrol and Diabetic + Glyclazide treated diabetic rats were compared with diabetic rats.

Units: Aldose reductase – nmoles/min/mg protein; Sorbitol dehydrogenase – Units/g protein; Glyoxalase-I – nmoles/min/mg protein; Serum AGEs – µg/mL.

TABLE 4. Effect of resveratrol on the levels of lipid peroxides, hydroperoxides and protein carbonyls in the kidney tissues of control and experimental groups of rats

Groups	Lipid peroxides	Hydroperoxides	Protein carbonyls
Control	1.18 ± 0.10	54.24 ± 1.94	4.49 ± 0.19
Control + Resveratrol	1.27 ± 0.07	54.59 ± 1.78	4.45 ± 0.17
Diabetic control	3.83 ± 0.19 <sup>a</sup>	82.81 ± 2.30 <sup>a</sup>	15.41 ± 0.86 <sup>a</sup>
Diabetic + Resveratrol	1.99 ± 0.07 <sup>b</sup>	64.50 ± 2.88 <sup>b</sup>	8.65 ± 0.40 <sup>b</sup>
Diabetic + Glyclazide	2.02 ± 0.09 <sup>b</sup>	62.29 ± 1.50 <sup>b</sup>	7.58 ± 0.36 <sup>b</sup>

Values are given as mean ± SEM for groups of six rats in each. One way ANOVA followed by post hoc test LSD. Values are statistically significant at  $p < 0.05$ . <sup>a</sup> Diabetic rats were compared with control rats; <sup>b</sup> Diabetic + Resveratrol and Diabetic + Glyclazide treated diabetic rats were compared with diabetic rats.

Units: Lipid peroxides – mmoles of TBARS/100 g tissues; hydroperoxides – mmoles/100 g tissues; Protein carbonyls – nmoles/mg protein.

TABLE 5. Effect of resveratrol on the levels of kidney non-enzymatic antioxidants such as vitamin C, vitamin E and GSH in control and experimental groups of rats

Groups	Vitamin C ( $\mu\text{g}/\text{mg}$ protein)	Vitamin E ( $\mu\text{g}/\text{mg}$ protein)	GSH ( $\text{mg}/100$ g wet tissue)
Control	$1.34 \pm 0.05$	$1.03 \pm 0.04$	$33.52 \pm 1.53$
Control + Resveratrol	$1.42 \pm 0.03$	$1.01 \pm 0.03$	$31.84 \pm 1.21$
Diabetic control	$0.43 \pm 0.02^{\text{a}}$	$0.47 \pm 0.02^{\text{a}}$	$20.57 \pm 0.88^{\text{a}}$
Diabetic + Resveratrol	$0.94 \pm 0.03^{\text{b}}$	$0.93 \pm 0.02^{\text{b}}$	$29.22 \pm 1.02^{\text{b}}$
Diabetic + Glyclazide	$0.97 \pm 0.02^{\text{b}}$	$0.85 \pm 0.03^{\text{b}}$	$29.28 \pm 0.94^{\text{b}}$

Values are given as mean  $\pm$  SEM for groups of six rats in each. One way ANOVA followed by post hoc test LSD. Values are statistically significant at  $p < 0.05$ . <sup>a</sup> Diabetic rats were compared with control rats; <sup>b</sup> Diabetic + Resveratrol and Diabetic + Glyclazide treated diabetic rats were compared with diabetic rats.

TABLE 6. Effect of resveratrol on the activities of SOD, catalase, GPx, GST and GR in renal tissues of control and experimental groups of rats

Groups	SOD	Catalase	GPx	GST	GR
Control	16.11 ± 0.93	40.42 ± 1.19	7.21 ± 0.19	6.26 ± 0.14	31.89 ± 1.68
Control + Resveratrol	17.75 ± 0.86	41.97 ± 1.67	7.58 ± 0.18	6.11 ± 0.18	32.99 ± 1.29
Diabetic control	9.82 ± 0.67 <sup>a</sup>	22.41 ± 1.36 <sup>a</sup>	4.07 ± 0.12 <sup>a</sup>	2.20 ± 0.11 <sup>a</sup>	9.78 ± 0.65 <sup>a</sup>
Diabetic + Resveratrol	14.25 ± 0.51 <sup>b</sup>	32.85 ± 1.16 <sup>b</sup>	6.44 ± 0.17 <sup>b</sup>	5.31 ± 0.17 <sup>b</sup>	23.74 ± 1.30 <sup>b</sup>
Diabetic + Glyclazide	13.87 ± 0.48 <sup>b</sup>	33.01 ± 1.01 <sup>b</sup>	6.69 ± 0.19 <sup>b</sup>	5.37 ± 0.18 <sup>b</sup>	23.45 ± 1.56 <sup>b</sup>

Values are given as mean ± SEM for groups of six rats in each. One way ANOVA followed by post hoc test LSD. Values are statistically significant at  $p < 0.05$ . <sup>a</sup> Diabetic rats were compared with control rats; <sup>b</sup> Diabetic + Resveratrol and Diabetic + Glyclazide treated diabetic rats were compared with diabetic rats.

Activity is expressed as: 50% of inhibition of epinephrine autoxidation/min for SOD;  $\mu$ moles of hydrogen peroxide decomposed/min/mg protein for catalase;  $\mu$ moles of glutathione oxidized/min/mg protein for GPx; units/min/mg protein for GST;  $\mu$ moles of DTNB-GSH conjugate formed/min/mg protein for GR.

#### Research Highlights

- ✓ Resveratrol protects diabetic kidney from hyperglycemia mediated oxidative stress.
- ✓ This effect is facilitated by normalization of renal proinflammatory cytokines.
- ✓ Resveratrol regulates antioxidant enzymes activities through Nrf2-Keap signaling.
- ✓ Resveratrol provides a new strategy for diabetic nephropathy treatment.