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Research Article
Resveratrol Ameliorates Kidney Injury and Fibrosis Secondary to Diabetes in Association with Inflammation and Nitrosative Stress Inhibition in Rats

Maha A. Momenah

Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia

Abstract

Background and Objective: Nephropathy secondary to diabetes is a severe disease that can advance to end-stage renal failure leaving kidney transplant as the only available method of treatment. This study sought to determine whether the plant polyphenolic compound, resveratrol (RSV) can inhibit diabetes-induced kidney fibrosis axis, inflammation/nitrosative stress/collagen in rats. **Materials and Methods:** Type 2 Diabetes Mellitus (T2DM) was established in rats using a blend of a high-fat diet and a single injection of streptozotocin. Whereas, the protective group of rats was pretreated with RSV suspension (30 mg kg^{-1}) followed by diabetes induction and continued on resveratrol until the scarification day for all the groups, at 12 week. **Results:** Hyperglycemia, weight loss, high levels of blood creatinine and urea and substantial kidney collagen fibers deposition confirmed diabetic nephropathy development. Also, diabetes significantly ($p<0.0001$) induced blood and kidney tissue protein expression of (1) Inflammation, Tumour Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) and (2) The nitrosative stress marker, Inducible Nitric Oxide Synthase (iNOS) enzyme using immunohistochemistry. All the above-mentioned parameters were inhibited by RSV. Furthermore, a significant correlation was detected between inflammation/iNOS/collagen axis and kidney injury biomarkers. **Conclusion:** Induction of T2DM for 10 weeks in rats develops nephropathy associated with the induction of inflammation and nitrosative stress, which is protected by resveratrol. This may suggest a likely therapeutic potential in diabetic nephropathy.

Key words: Diabetic nephropathy, iNOS, inflammation, urea, creatinine, kidney fibrosis, resveratrol

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Corresponding Author: Maha A. Momenah, Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia

Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The rapid rise in obesity worldwide due to adopting a Western-style diet rich in highly processed food is the main cause of increasing non-communicable diseases like diabetes, cancer and heart disease among the genetically vulnerable people that claim around thirty-five million death every year[1]. The link between insulin resistance and obesity associated with the upregulation of inflammation and oxidative stress is well documented[2] and the occurrence of obesity among adults in the USA with diagnosed type 2 diabetes mellitus was 54.8% between 1999-2002[3]. Obesity and increased body mass index are also linked to proteinuria, glomerulopathy, diabetic nephropathy and renal failure[4-6]. However, maintaining a normal body weight can reduce the risk of renal failure in men but not in women[4]. Furthermore, in the USA, diabetic nephropathy is the cause of nearly 44% of patients on dialysis due to renal failure[7].

In both, type 1 and type 2 diabetes mellitus-induced kidney injury, glomerular blood vessel damage occur which reduces the glomerular filtration rate and increases proteinuria as well as hypertension[8]. However, not all diabetic patients will develop nephropathy and controlling glycemia and dyslipidemia would decrease the possibility of developing diabetic nephropathy[8]. Oxidative stress, inflammation and renin-angiotensin system activation are involved in diabetic nephropathy development via the thickening of the glomerular basement membrane, expansion of mesangial cells, proteinuria and hypertension[9,10]. Indeed, renal infiltration of inflammatory cells induced kidney injury and iNOS expression in kidney cells correlated with glomerular injury[11,12]. The inflammatory marker TNF-α was reported to fall upstream of iNOS in macrophages from patients with the diabetic neuropathy[13].

The antioxidant and anti-inflammatory plant compound resveratrol (RSV)[14,15] was proven to be effective in the treating/protecting against kidney disease in humans and animals, for example, (1) RSV protected against the induction of oxidative stress (ROS) caused by hyperglycemia in glomerular mesangial tissue culture cells[16], (2) RSV inhibited mouse renal tubular cells against apoptosis and injury induced by the chemotherapy drug, cisplatin[17], (3) RSV ameliorated renal fibrosis in obese mice with severe hyperglycemia via the regulation of AMPK/NOX4/ROS axis[18], (4) RSV proposed to delay kidney ageing via different pathways including the augmentation of telomerase activity[19] and (5) RSV at a dose of 250 mg per day for 3 months improved glycated haemoglobin and blood pressure in diabetic patients[20].

Therefore, this study anticipated that diabetes can induce inflammation/nitrosative stress axis mediated kidney fibrosis and that resveratrol can inhibit these parameters in an animal model of diabetic nephropathy.

MATERIALS AND METHODS

Study area: This study was carried out at the Research Centre, College of Medicine, King Khalid University and Princess Nourah Bint Abdulrahman University, Saudi Arabia from April, 2021 to 2022.

Animals: Albino rats were used in this project and performed experiments following recommendations for the care and handling of animals approved by King Khalid University. Therefore, rats (170-200 g) were kept in cages and housed in a clean facility that provides controlled humidity and a stable room temperature (22°C), as well as 12 hrs light/dark cycles. In addition, free access to water and food was permitted for these rats.

Experimental design: After 7 days of acclimatization, rats (n = 24 total) were separated into three groups. These included (1) The control group, (2) The diabetic group (T2DM), diabetes was established using a blend of a high carbohydrate and fat diet (HCFD) and a single injection of streptozotocin as reported before[21] and (3) The protective group (RSV+T2DM), diabetes was induced in this group similar to the previous group but the treatment with RSV (30 mg kg^{-1}, orally, daily) started from day 1, before diabetes induction and continued until 12 week. All rats were culled at week 12. Then, blood was collected under anaesthesia and animals were culled and organs were harvested.

Immunohistochemistry of Inducible Nitric Oxide Synthase (iNOS) and evaluation of disease phenotype: The iNOS immunostaining and sirius red staining were accomplished as previously described[22]. Paraffin blocks of kidney tissue were prepared from all rat groups using standard methods and sections (5 µm) were processed for immunohistochemistry and sirius red methods. Kidney sections were incubated with anti-iNOS (Abcam, Cambridge, UK) overnight at 4°C. The sections were then incubated with the secondary antibody at room temperature for a half-hour and counter-stained with Meyer's hematoxylin. To quantify the degree of kidney fibrosis, sirius red staining for collagen deposition in kidney sections prepared from all three animal groups was performed. Quantification of iNOS collagen (areas %) was achieved using an image analyzer.

Estimation of blood glucose, TNF-α, IL-6, urea and creatinine: Blood levels of glucose (Randox reagent kit, Sigma-Aldrich) and urea and creatinine (BioAssay System, USA) were measured in all rats' groups using colorimetric methods. Blood levels of TNF-α and IL-6 were estimated in rats using ELISA kits supplied by Invitrogen, Waltham, MA, USA. All procedures were accomplished according to the manufacturer's guidelines.

Statistical analysis: Data are presented as the Mean±SD. SPSS version 10.0 was used to analyze the generated data. One-way ANOVA followed by Tukey's *post hoc* test was used to perform the statistical comparisons. Results are significant if $p \leq 0.05$.

RESULTS

Resveratrol (RSV) ameliorates biomarkers of inflammation and renal injury induced secondary to diabetes in rats: Upregulation of renal and systemic inflammation post diabetes is well documented as well as inflammation induces renal injury. Therefore, blood levels of inflammation such as TNF-α and IL-6 and the renal injury indicators, urea and creatinine with and without RSV incorporation were assessed (Fig. 1). RSV significantly ($p \leq 0.0165$) inhibited diabetes-induced glucose (Fig. 1a), TNF-α (Fig. 1b), IL-6 (Fig. 1c), urea (Fig. 1d) and creatinine (Fig. 1e). A sharp decrease in body weight caused by diabetes over 10 weeks, was significantly ($p<0.0001$) resorted by RSV when compared with the control group (Fig. 1f). However, only blood urea was completely inhibited to levels comparable to control (Fig. 1d).

Resveratrol (RSV) ameliorates kidney tissue levels of nitrosative stress (iNOS) induced secondary to diabetes in rats: In cell signalling, inflammatory biomarkers are located upstream of iNOS. Therefore, kidney tissue levels of iNOS protein expression were assessed in all rats groups (Fig. 2). Representative image from tissue sections obtained from the control rats shows no iNOS expression (Fig. 2a). Whereas, renal tubules showed strong iNOS positive immunostaining in sections prepared from the diabetic untreated rats (Fig. 2b). RSV treatment substantially reduced the expression of kidney iNOS in the protective group (Fig. 2c). A quantitative assessment of iNOS positive cells showed a significant ($p<0.0001$) increase by diabetes, which was protected ($p<0.0001$) by RSV to levels still significant to control (Fig. 2d). This means partial protection by RSV.

Resveratrol (RSV) ameliorates kidney fibrosis (collagen) induced secondary to diabetes in rats: The iNOS is a known contributor to tissue fibrosis. Therefore, kidney fibrosis measured as collagen deposition was evaluated in all rats

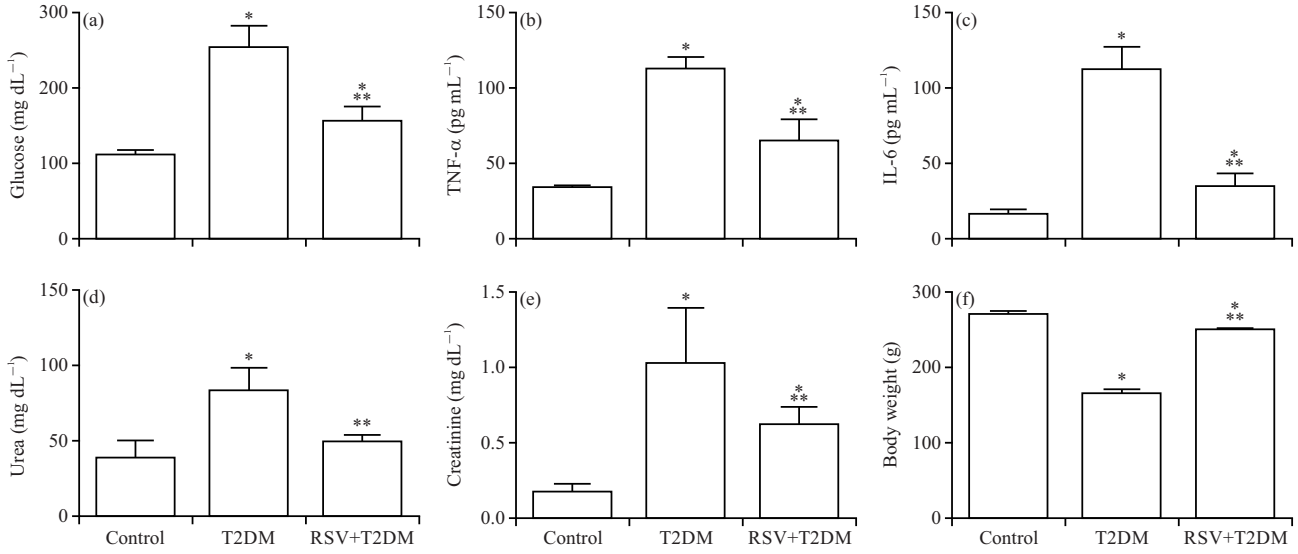

Fig. 1(a-f): Diabetes induces inflammation and kidney injury biomarkers and is inhibited by resveratrol (RSV), (a) Blood levels of glucose, (b) TNF-α, (c) IL-6, (d) Urea, (e) Creatinine and (f) Body weight were assessed in all rats' groups at 12 week

All p values are significant, *$p \leq 0.0108$ versus control and **$p \leq 0.0165$ versus T2DM, TNF-α: Tumour necrosis factor-alpha, T2DM: Type 2 diabetes mellitus, IL-6: Interleukin-6 and X-axis of a-f: Groups

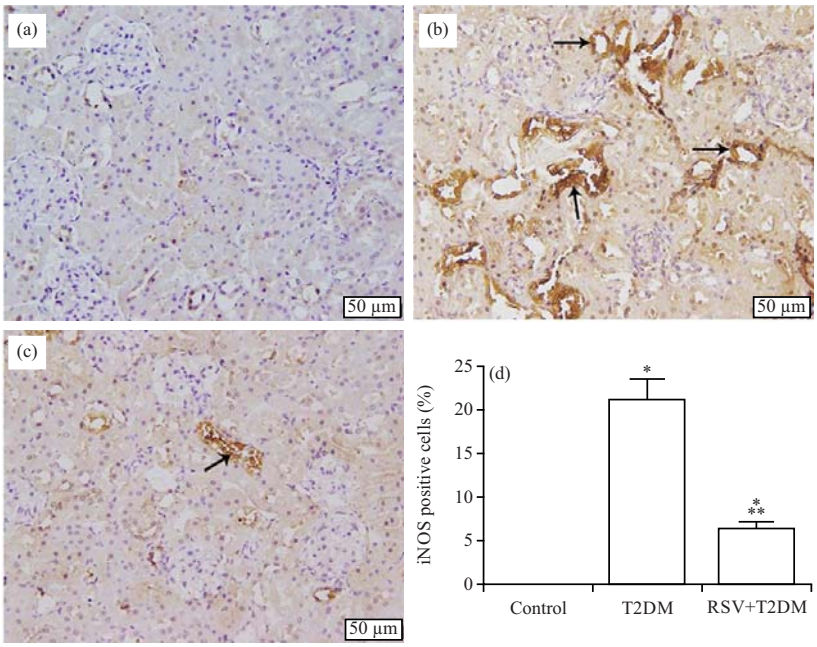

Fig. 2(a-d): Diabetes induces kidney expression of iNOS, which is inhibited by resveratrol (RSV), (a) Images (x200) of iNOS+ve immunostained kidney sections from the control, (b) T2DM, (c) RSV+T2DM rats' groups are illustrated and (d) Percentage of iNOS positive immunostaining kidney sections is shown in all rat groups

All p values are significant, *p<0.0001 versus control and **p<0.0001 versus T2DM, arrows point to the strong and weak +ve immunostaining cells in b and c, respectively, iNOS: Inducible nitric oxide synthase and X-axis of the D part: Groups

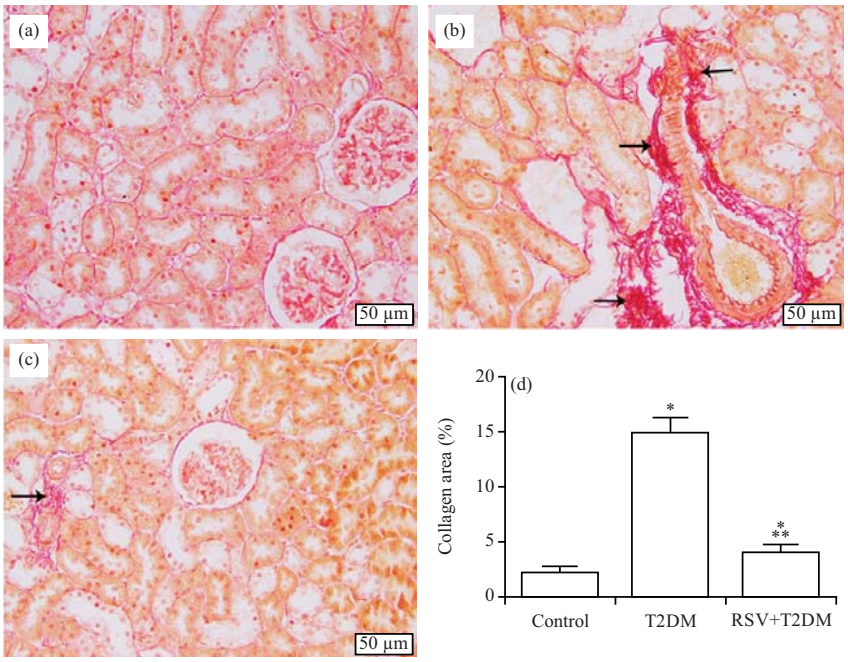

Fig. 3(a-d): Diabetes induces kidney collagen expression is inhibited by resveratrol (RSV), (a) Images (x200) of collagen+ve sirius red staining kidney sections from the control, (b) T2DM, (c) RSV+T2DM rats' groups are illustrated and (d) Percentage of fibrosis deduced from the sirius red staining kidney sections is shown in all rats' groups

All p values are significant, *p≤0.0074 versus control and **p<0.0001 versus T2DM, arrows point to the strong and weak positive collagen deposition in b and c, respectively, T2DM: Type 2 diabetes mellitus and X-axis of the D part: Groups

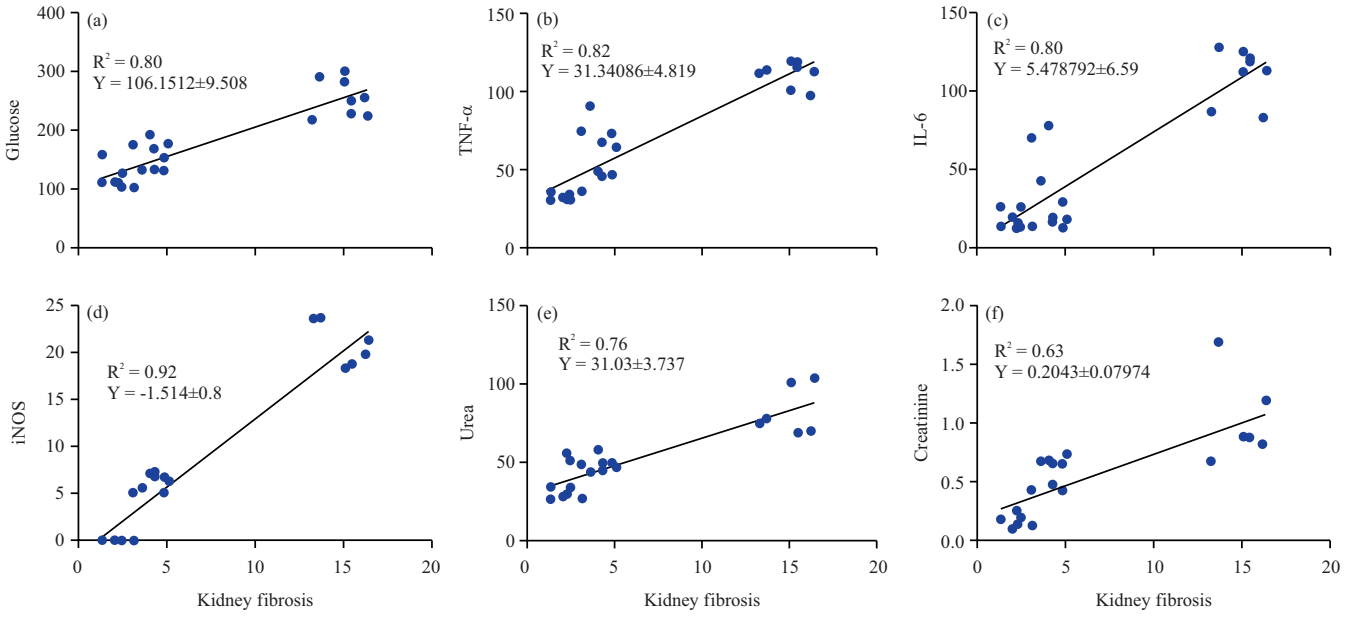

Fig. 4(a-f): Kidney fibrosis score correlates with diabetic nephropathy, levels of fibrosis (collagen deposition) in kidney tissue were determined in all rats at the end of the study and a positive correlation ($p<0.0001$) was observed between kidney fibrosis versus, (a) Glucose, (b) TNF-α, (c) IL-6, (d) iNOS, (e) Urea and (f) Creatinine

TNF-α: Tumour necrosis factor-alpha, IL-6: Interleukin-6, iNOS: Inducible nitric oxide synthase and X-axis of a-f: Kidney fibrosis score

(Fig. 3). Sirius red-stained sections of the control group (Fig. 3a) showed a weak collagen deposition compared to intense collagen deposition in the renal interstitium and the surrounding blood vessels (Fig. 3b). RSV treatment for 12 weeks substantially ameliorated kidney fibrosis (Fig. 3c). A quantitative assessment of kidney fibrosis revealed that diabetes-induced significant ($p<0.0001$) increase in collagen fibers, which was protected by RSV to levels still significant ($p\leq0.0074$) to control (Fig. 3d). This also means partial protection by RSV.

Correlation between kidney fibrosis score and diabetic nephropathy markers: The correlation between collagen deposition (fibrosis) score and biomarkers of diabetic nephropathy, glucose, inflammation, iNOS, urea and creatinine was determined. This links inflammation/iNOS axis with kidney fibrosis as well as showing the potential therapeutic value of RSV. Kidney fibrosis score demonstrated a significant ($p<0.0001$) positive correlation with g 0.898 (Fig. 4a), TNF-α ($r = 0.909$) (Fig. 4b) 0.898 (Fig. 4c), iNOS 0.959 (Fig. 4d) 0.877 (Fig. 4e) and creatinine 0.795 (Fig. 4f).

DISCUSSION

This article investigated the inflammation/iNOS axis mediated fibrosis in a diabetic nephropathy rat model with and without the incorporation of resveratrol (RSV). In this rat model, the hypothesis that RSV can inhibit kidney inflammation/iNOS/collagen axis was examined 10 weeks after diabetic induction. The data presented in this study demonstrated after 10 weeks following the STZ injection, the induction of glycemia, inflammation (TNF-α and IL-6), nitrosative stress (iNOS), biomarkers of renal injury (urea and creatinine) and kidney fibrosis (collagen deposition), which were protected by RSV (Fig. 1-3). In addition, the data obtained from all groups of rats showed a correlation between kidney fibrosis score and the above-mentioned diabetic nephropathy markers (Fig. 4), which further support an association between RSV and amelioration of kidney inflammation/iNOS/collagen axis mediated kidney in jury (Fig. 5). Therefore, the working hypothesis was supported by the collected data shown in this report.

Diabetic nephropathy is reported in both, human and animal models[23,24] as demonstrated by hyperglycemia, microalbuminuria, inflammation and fibrosis and increased urea and creatinine blood levels[9,23,24]. In addition, infiltration of inflammatory cells such as macrophages into the kidney cortex induced glomerulonephritis and macrophages induced proliferation of mesangial cells and proteinuria in rats[12]. These reports were in agreement with this study displaying an increase in all the above-mentioned parameters. Furthermore, induction of insulin-dependent diabetes in mice

Fig. 5: Proposed model for diabetic nephropathy which appears to be ameliorated by resveratrol
T2DM: Type 2 diabetes mellitus, RSV: Resveratrol and iNOS: Inducible nitric oxide synthase

caused the upregulation of inflammatory cytokines and iNOS expression in the pancreatic islets and inhibitor of iNOS blocked kidney fibrosis in mice established by unilateral ureteral obstruction[25,26] agree with the data shown in Fig. 1 and 2.

The anti-inflammatory effect of RSV delayed polycystic kidney disease progression[27] RSV is also suggested to inhibit kidney fibrosis in chronic kidney disease[28] as well as beneficial effects in patients on hemodialysis[29] and inhibiting diabetic nephropathy via inhibiting the oxidative stress-induced damage on renal tubules[30]. These reports were also in agreement with this study demonstrating that RSV inhibited inflammation, nitrosative stress and kidney fibrosis.

CONCLUSION

Taken together, the data presented in this work support the conclusion that induction of nephropathy secondary to T2DM in rats over 10 weeks caused the augmentation of inflammation, nitrosative stress, kidney injury and renal fibrosis, which appeared to be inhibited by the polyphenolic anti-inflammatory compound, resveratrol. In addition, an association was demonstrated between kidney fibrosis and these parameters.

SIGNIFICANCE STATEMENT

This report represents an important contribution to the study of diabetic nephropathy induced 10 weeks following the induction of type 2 diabetes mellitus in an animal model since it demonstrates the activation of the inflammation/nitrosative stress axis-mediated kidney injury and fibrosis, which appeared to be inhibited by the plant-derived polyphenolic compound resveratrol. This may offer therapeutic potential in patients with kidney injury secondary to diabetes.

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