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Effects of pioglitazone and rosuvastatin on inflammatory gene expression and mitigation of diabetic nephropathy

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ABSTRACT: Egypt is the eighth-leading country in the prevalence of diabetes mellitus (DM). It was estimated that more than ten million adults would suffer from DM in Egypt in 2022. Diabetic nephropathy (DN) is one of the complications of DM, which can lead to end-stage renal disease (ESRD). There is limited information on the efficacy of pioglitazone and/or rosuvastatin in diabetic kidney diseases (DKD). In this study, 40 male rats were divided into two groups: the control group (8 rats) and the diabetic group (32 rats), in which rats were fed a high-fat diet (HFD) for 4 weeks and then injected intraperitoneally (IP) with streptozotocin (STZ). The diabetic group was then subdivided into four equal groups as follows: Group 1: diabetic non-treated; Group 2: diabetic + treated with pioglitazone orally at a dose of 10 mg/kg once daily for 4 weeks; Group 3: diabetic + treated with rosuvastatin orally at a dose of 10 mg/kg once daily for 4 weeks; and Group 4: diabetic + treated with pioglitazone and pioglitazone orally at a dose of 10 mg/kg for each of them once daily for 4 weeks. At the end of the experiment (8 weeks), blood samples were taken to study their effects on the diabetic kidney by evaluating kidney function tests. The left kidney was taken to detect the gene expression of inflammatory markers such as tumor necrosis factor-α (TNF-α) and interleukin (IL-1β), as well as CYP 2E1, and the right kidney was taken to detect histopathological changes in renal tissues.

KEYWORDS: Diabetes mellitus, Diabetic nephropathy, Gene expression

1. Introduction

Type 2 diabetes mellitus (T2DM) is mainly caused by a combination of insulin resistance and inadequate insulin secretion [1]. It is characterized by chronic hyperglycemia, which is associated with an increase TNF-α production and oxidative stress leading to the development of disabling and life-threatening health complications, the most prominent of which is DN[2, 3, 4]. A typical microvascular consequence of diabetes, DKD is characterized by a progressive deterioration of albuminuria. Individuals with DKD are more likely to develop cardiovascular diseases, ESRD, and an increased risk of renal dysfunction (elevation in serum creatinine, urea, and uric acid and reduction in glomerular filtration rate)[5]. Pioglitazone is an insulin-sensitizing thiazolidinedione (TZD) agent that is widely used in T2DM treatment. It acts by binding to the nuclear PPAR-γ, which leads to increased insulin sensitivity in the liver and peripheral tissues, improving glycemic control with no increase in insulin secretion [6]. It also improves serum lipid profiles through action at PPARα and reduces the risk of CVD in patients with T2DM [7]. TZDs may have anti-inflammatory qualities in addition to helping to reduce insulin resistance and regulate blood sugar levels [8]. In addition, pioglitazone reduces TNF-α (increased expression is associated with DM/obesity) and improves metabolic abnormalities in Wistar fatty rats [9]. Statins are the most commonly used lipid-lowering medications and the first choice for the treatment of diabetic dyslipidemia to decrease the risk of adverse CVD. The principal therapeutic effect of statin medications is a reduction in levels of circulating atherogenic lipoproteins as a result of competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme

A (HMG-CoA) reductase, mainly in the liver [10]. Rosuvastatin is a recent statin and is widely used for the prevention of atherosclerotic CVD. Compared with other commonly used statins, rosuvastatin is a more potent inhibitor of HMG-CoA reductase and has a high degree of selectivity for effect in liver cells compared with a range of non-hepatic cells, so it has the highest lipid-lowering effect. In addition, it has relatively low drug-drug interaction as compared to other statins because it undergoes relatively little metabolism by the hepatic CYP3A4 system. It has a relatively long elimination half-life and lower rates of statin-related adverse events [11].

Some studies have shown that statins therapy is associated with improved renal function in patients with DM and those with chronic renal insufficiency and CVD [12].

Diabetes mellitus is frequently associated with hyperlipidemia. Management of lipid disorders reduces vascular complications in diabetic patients. Thus, most of the patients suffering from diabetic dyslipidemia need antidiabetic drugs such as pioglitazone and hypolipidemic drugs such as rosuvastatin. Accordingly, the current work is aimed at evaluating possible drug interactions between pioglitazone and rosuvastatin in diabetic rats through their effect on DKD.

2. Materials and methods

2.1. Chemicals and kits

Streptozotocin was derived from Sigma-Aldrich, USA. Sodium citrate and citric acid were acquired from SD Fine-Chem Ltd., Mumbai, India. Pioglitazone was obtained from Unipharma Company, Egypt, in the form of white tablets (30 mg), and it was suspended in distilled water. Rosuvastatin calcium (Crestor) was obtained from Astra Zeneca, Egypt, in the form of pink tablets (20 mg), and it was dissolved in distilled water. Isoflurane 1% was bought from Hospira, Inc., USA. The FreeStyletyle Optium glucometer was obtained from Witney, Oxon, UK, with its specific glucose strips. Creatinine and uric acid kits were purchased from Spinreact, Spain, and urea kits were acquired from Diamond Diagnostic, Egypt. RNeasy mini extraction kits were bought from Qiagen, Germany.

2.2. Experimental animals and design

This experiment was carried out according to the guidelines of the Institutional Review Board, Faculty of Medicine, Assiut University Committee, Egypt, and Approval (04-2023-100075). Fourty adult male Wister albino rats were purchased from the animal house, Faculty of Veterinary Medicine, Assuit University, weighing between 120-150 grams and aged 7-8 weeks. They were kept in metal cages and housed in a well-ventilated room throughout the study and acclimatized for two weeks at a temperature of 18–24°C with 12 hours of light and darkness on a normal feed diet and water Ad Libitum.

2.3. Induction of DM:

The experimental diabetes in rats was induced by HFD and a low dose of STZ. HFD contained 58% fat, 25% protein, and 17% carbohydrate. Rats were fed with HFD for 28 days, followed by fasting overnight. Then, STZ was dissolved in a 0.1 M cold sodium citrate buffer (pH 4.5) [13], and was given IP at a dose of 35 mg/kg B.W. [14]. After diabetes induction, rats were allowed to drink a 0.5% glucose solution during the firest 24 hours to overcome severe hypoglycemia [15]. After 3 days, the blood glucose of each animal was measured using the digital glucometer. Rats with fasting blood glucose of 250 mg/dl and above were considered diabetic and selected for the experiment [16].

2.4. Animal groups

Rats were randomly divided into two groups: the control group (8 rats), in which rats were injected with an equivalent volume of 0.1 M citrate buffer, pH 4.5, as the STZ vehicle, and the diabetic group (32 rats), in which rats were fed HFD for 4 weeks and then injected IP with STZ. The diabetic group was then subdivided into four equal groups as follow:

- **Group 1:** diabetic, non-treated

- **Group 2:** diabetic and treated with pioglitazone orally at a dose of 10 mg/kg once daily for 4 weeks.
- **Group 3:** diabetic and treated with rosuvastatin orally at a dose of 10 mg/kg once daily for 4 weeks.
- **Group 4:** diabetic and treated with pioglitazone and rosuvastatin orally at a dose of 10 mg/kg for each of them once daily for 4 weeks.

At the end of the experimental period, the animals, after overnight fasting, were anesthetized with isoflurane. Blood samples were collected at the medial canthus of the retro-orbital plexus using a capillary tube. Rats were then sacrificed, and kidney tissue samples were collected under appropriate laboratory standards. Serum was separated by centrifugation at 3000 rpm for 15 min. and stored at -20°C. Kidney tissue was removed. The left kidney was stored at -80°C for molecular assays. Moreover, the right kidney was stored for histopathological examination in 10% neutral buffered formalin.

2.5. *Determination of kidney function tests:*

Serum creatinine, urea, and uric acid levels were determined using creatinine, urea, and uric acid enzymatic colorimetric kits according to the manufacturer's protocol [17].

2.6. *Histopathological examination of renal tissue:*

The right kidney was taken and immediately fixed in 10% neutral buffered formalin for 24 hours. Then, the samples were dehydrated by increasing rates of ethyl alcohol. The dehydrated samples were cleared in xylol for 6 hours. The specimens were then blocked in hard paraffin and cut into sections at a thickness of about 5 microns. After that, they were stained with haematoxylin and eosin (H&E) for examination by a light microscope [18]. Histological scoring was performed as follows:

- No change was counted as 0.
- Mild changes was scored as 1.
- Moderate changes was scored as 2.
- A score of 3 was given for severe changes

2.7. *Gene expression evaluation:*

Total RNA was extracted from the renal tissue samples in accordance with the manufacturer's instructions using the RNeasy mini extraction kit. The spectrophotometry was employed at wavelength 260 to determine the concentration and the 260:280 ratio to select the pure samples that were in the range of 1.8 and 2.0. After then, DNase I was used to clean up the DNA contamination (Fermentas, Lithuania), and complementary DNA (cDNA) was produced using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) in line with the manufacturer's instructions. The primer sets for measuring the mRNA levels of particular genes were generated using the Rattus norveicus sequences present in GenBank (Table 1). The primer3 tool was used to build the primers. SYBR Green PCR Master Mix was used in real-time PCR analysis to assess the relative expression of the selected genes (Thermo Scientific Cat number: 4309155). Applied Biosystems' ABI Prism StepOnePlus Real-Time PCR System as directed by the manufacturer For each sample, the PCR reactions were conducted twice. The expression levels were normalized for the housekeeping gene beta-actin. The data on gene expression were analyzed using the DDCt method [19].

2.8. *Statistical analysis*

Data analysis was performed using Graph Pad Prism 8 (Graph Pad Software Inc., San Diego, USA). A P-value of < 0.05 was adopted as statistically significant and analyzed by one-way analysis of variance (ANOVA). The data were presented as means ± standard errors of means (SEM).

Table 1: Rattus Norveicus sequences present in GenBank

Gene	Sense	Antisense	Accession No.
IL-1β	TTGAGTCTGCACAGTTCCCC	GTCCTGGGGAAGGCATTAGG	NM_031512.2
TNF-α	ACACACGAGACGCTGAAGTA	GGAACAGTCTGGGAAGCTCT	NM_012675.3
Cyp2E1	ATGAGTTTTCTGGACGGGGG	TTTGGATGCGGGCCTCATTA	NM_031543.2

3. Results

3.1. *Effect of pioglitazone, rosuvastatin and their combination on kidney function tests of diabetic rats:*

Serum creatinine, urea, and uric acid were significantly increased in diabetic rats in comparison with control rats. On the other hand, serum creatinine, urea, and uric acid levels in diabetic rats treated with pioglitazone, rosuvastatin, or their combination were significantly decreased in comparison with untreated diabetic rats. These results are given in (Table 2) and (Figs. 1 to 3)

Effect of, pioglitazone, rosuvastatin and their combination on renal inflammatory status

The results showed that, TNF-α and IL-1β was significantly increased in the kidney of diabetic rats in comparison with control rats. On the other hand, diabetic rats treated with pioglitazone, rosuvastatin or their combination orally for 28 consecutive days elicited high significant decrease in renal TNF-α and IL-1β. These results are given in (Table 3 and Figs. 4 and 5).

Effect of pioglitazone, rosuvastatin and their combination on transcript level of CYP2E1 in kidney of diabetic rats:

Renal CYP2E1 was significantly increased in diabetic rats in comparison with control rats. However, diabetic rats that received pioglitazone, rosuvastatin, or their combination for 4 weeks revealed a significant decrease when compared with diabetic rats that received no treatment. These results are given in (Table 3 and Fig. 6)

Histopathological observations of renal tissue:

Different histopathological lesions were recorded in Table 4, and the photomicrograph were illustrated from Figure Figs. 7 to 12

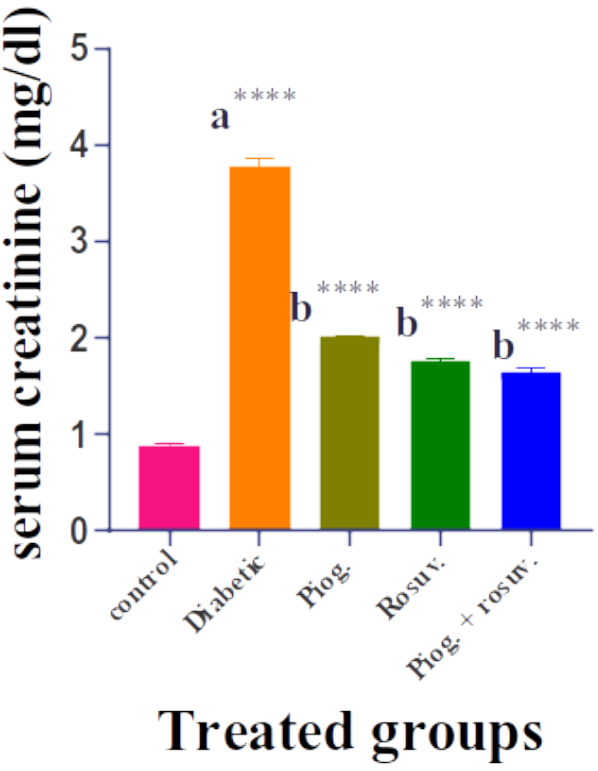


Figure 1: Effect of pioglitazone, rosuvastatin and their combination on serum creatinine of diabetic rats. Data represents the mean ± SEM of each group (n=8). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (**** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

Discussion

Type 2 diabetes is a serious metabolic disorder caused by abnormal carbohydrate metabolism, and closely associated with renal dysfunction [20]. In this study, HFD was used to induce insulin resistance, while a low dose of STZ was given to create a partial insulin deficiency in rats. The presence of partial insulin deficiency with insulin resistance mimics T2DM in humans [21]. Urea and creatinine are the end products of protein and creatine metabolism. Therefore, the increased level of creatinine coincides with muscle waste, a commonly observed condition in DM [22]. Further, an increase in uric acid level was observed, which has been implicated in insulin resistance. Muscle wasting as a result of protein glycation leads to increased release of purine, which is the chief source of uric acid formation in DM. Increased serum uric acid is responsible for oxidative stress and the excess generation of TNF-α, which is strongly associated with diabetes progression. The significantly increased urea, creatinine, and uric acid levels in diabetic rats demonstrated renal damage and metabolic alteration resulting

Table 2: Effect of pioglitazone, rosuvastatin and their combination on serum creatinine, urea and uric acid of diabetic rats.

	Control rats	Diabetic rats	Piog. in diabetic rats	Rosuv. in diabetic rats	Piog. + rosuv. in diabetic rats
Serum creatinine (mg/dl)	0.89 ± 0.02	3.78 ± 0.09 **a******	2.01±0.014 **b******	1.76±0.041 **b******	1.64 ± 0.045 **b******
Serum urea (mg/dl)	30.23± 1.181	65.89±2.485 **a******	47.31±1.312**b******	44.68±1.355**b******	42.81± 1.085 **b******
Serum uric acid (mg/dl)	2.71 ± 0.097	7.54 ± 0.15 **a******	5.9 ± 0.12 **b******	5.15 ± 0.15 **b******	5.025 ± 0.13 **b******

Data represents the mean ± SEM of each group (n=8). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (**** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

Table 3: Effect of pioglitazone rosuvastatin and their combination on transcript level of TNF-α, IL-1β and CYP 2E1 in kidney of diabetic rats

	Control	Diabetic rats	Piog. in diabetic rats	Rosuv. in diabetic rats	Piog.+ rosuv. in diabetic rats
Renal tumor necrosis factor-α	1 ± 00	5.4 ± 0.15 **a******	4.5 ± 0.2 **b****	4.76 ± 0.05 **b***	4.1 ± 0.08 **b*****
Renal interleukin-1β	1 ± 00	7.7 ± 0.33 **a******	5.8 ± 0.08 **b******	5.6 ± 0.09 **b******	4.3 ± 0.09 **b******
Renal cytochrome P2E1	1 ± 00	9.1 ± 0.07 **a******	7.5 ± 0.01 **b******	7.66 ± 0.22 **b******	6.3 ± 0.21 **b******

Data represents the mean ± SEM of each group (n=3). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

Table 4: Histopathological changes in kidney of different treated groups

Microscopic lesions	Control	Diabetic	Piog.	Rosuv.	Piog.+ rosuv.
vacuolar degeneration of tubular epith.	0	3	2	1	1
vascular congestion	1	4	2	1	1
Necrosis	0	2	0	0	0
tubular atrophy	0	2	0	0	0
injury of blood vessels intema	0	2	1	0	0
Interstitial inflamatory cells infiltration	0	2	1	1	1
Hemorrhage	0	4	2	1	1

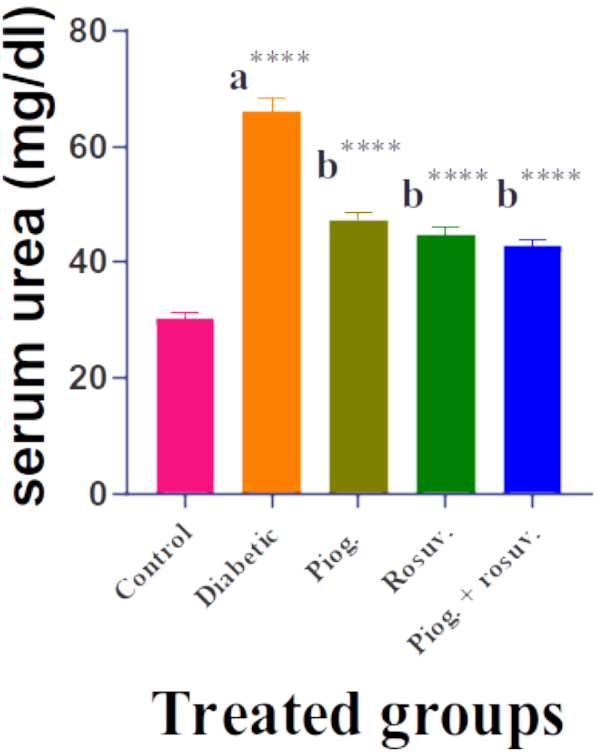


Figure 2: Effect of pioglitazone, rosuvastatin and their combination on serum urea of diabetic rats. Data represents the mean ± SEM of each group (n=8). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (**** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

from STZ-induced hyperglycemia [23]. At the end of four weeks following STZ injection, there was evidence of deranged kidney function in diabetic rats, as indicated by the elevated serum urea and creatinine levels as well as high serum uric acid levels. These findings were supported by the histopathological evidence, which showed vacuolar degeneration of tubular epithelium (3), vascular congestion (4), necrosis (2), tubular atrophy (2), injury of blood vessels intima (2), interstitial inflammatory cell infiltration (2), and hemorrhage (4) in the untreated diabetic rats. These findings agree with those of [24], who showed that HFD causes a cascade of clinical alterations in the kidney, involving tubular injury, renal inflammation, and cytokine expression, resulting in kidney disorder. It is well reported that DM induces renal corpuscular and tubular damage, including podocyte injury and renal corpuscular atrophy [21]. During this study, the kidneys of diabetic rats treated with pioglitazone revealed a decrease in kidney function tests and amelioration in histological damage. This comes in accordance with the findings of [25], that revealed that treatment with pioglitazone successfully counteracted the morphological alterations and renal function parameters in diabetic rats. In addition, no necrosis, tubular atrophy, and njury to the intima of blood

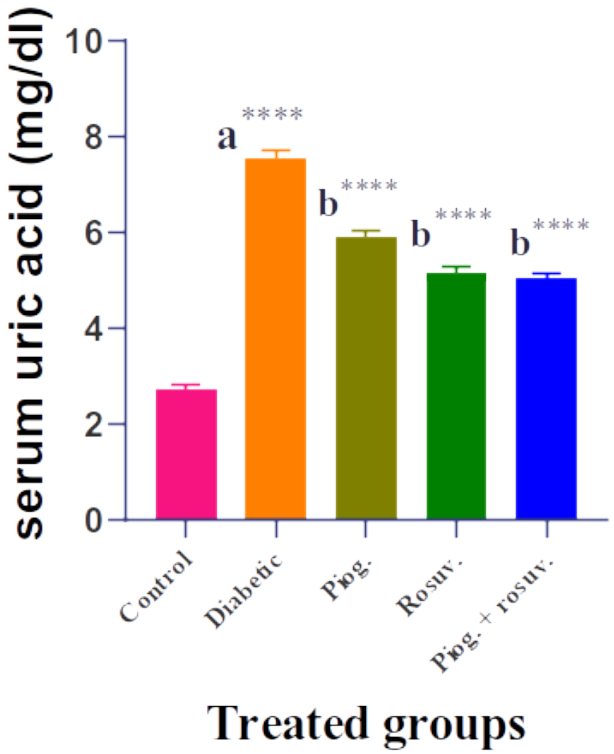


Figure 3: Effect of pioglitazone, rosuvastatin and their combination on serum uric acid of diabetic rats. Data represents the mean ± SEM of each group (n=8). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (**** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

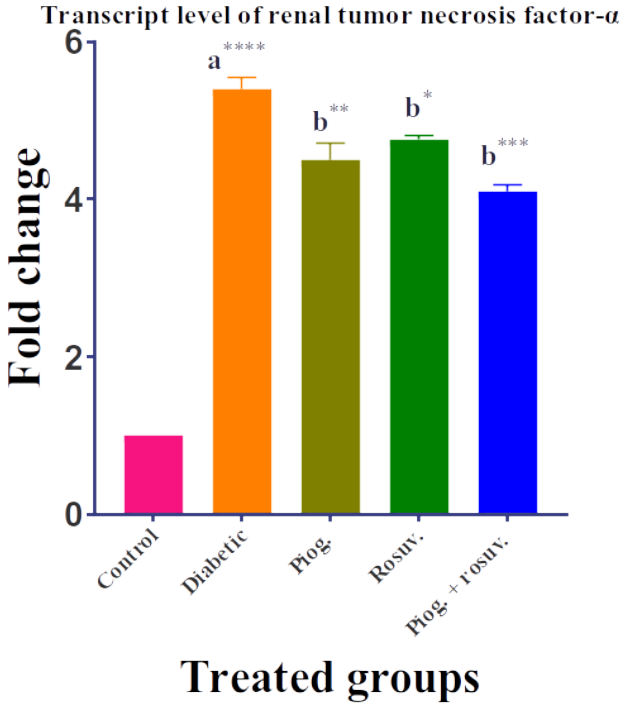


Figure 4: Effect of pioglitazone rosuvastatin and their combination on transcript level of TNF-α in kidney of diabetic rats.. Data represents the mean ± SEM of each group (n=3). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

vessels was seen with rosuvastatin treatment, but there was mild vacuolar degeneration of the tubular epithelium (1), vascular congestion (1), interstitial inflammatory cell infiltration (1) and hemorrhage (1). This result is identical to the [26] study, which detected significant histopathological improvements in the renal tissues of diabetic rats after six-week treatment with rosuvastatin. Furthermore, rosuvastatin has been reported to improve kidney function and ameliorate oxidative stress, independent of its lipid-modifying effects, in patients with DN [27]. This result is also in line with previous studies [28, 29], reporting that rosuvastatin improved kidney function and reduced oxidative stress independent of its effect on lipid levels in experimental diabetic nephropathy. To the contrary, despite all these beneficial effects, several studies indicated that rosuvastatin had harmful effects on the kidney. Administration of rosuvastatin was reported to cause acute interstitial nephritis in humans [30] and proximal tubule damage and renal toxicity in rats [31]. In addition, rosuvastatin caused renal damage associated with severe rhabdomyolysis, whether rosuvastatin was given alone [32, 33, 34], or in combination with colchicine [35], ticagrelor [36], or cocaine and heroin [37]. The harmful effects of rosuvastatin, which were reported in previously mentioned papers, might be because of the large dose of rosuvastatin used. The combination of pioglitazone and rosuvastatin provided a greater renoprotective effect than monotherapy. Similar outcomes were found in a study by [26] whose findings showed that rosuvastatin, when given in combination with an antioxidant such as coenzyme Q10, had considerably prevented experimentally induced diabetic nephropathy more than rosuvastatin alone. This may be due to decreased lipid peroxidation, TGF-β, TNF-α, MPO, and nitrite levels in kidney tissue.

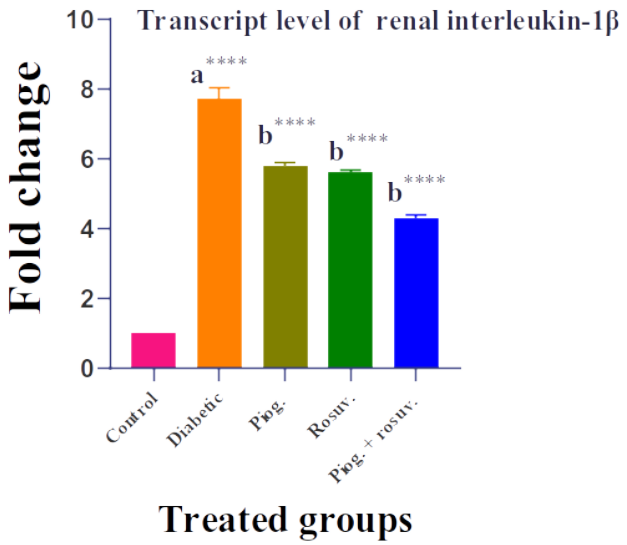


Figure 5: Effect of pioglitazone rosuvastatin and their combination on transcript level of IL-1β in kidney of diabetic rats. Data represents the mean ± SEM of each group (n=3). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (**** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

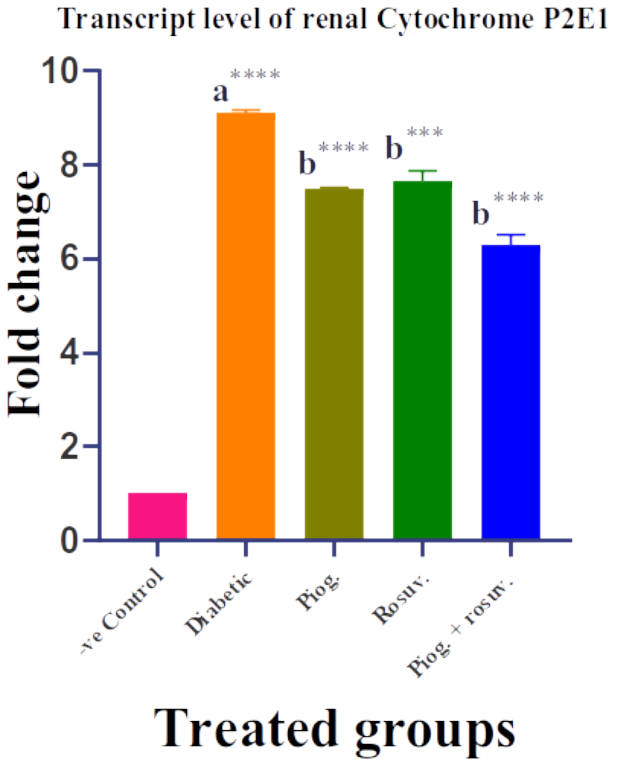


Figure 6: Effect of pioglitazone, rosuvastatin and their combination on transcript level of renal CYP2E1 of diabetic rats. Data represents the mean ± SEM of each group (n = 3). a: significant in comparison with control rats. b: significant in comparison with diabetic rats. P values (*** < 0.001, **** < 0.0001). Piog: pioglitazone and Rosuv: rosuvastatin.

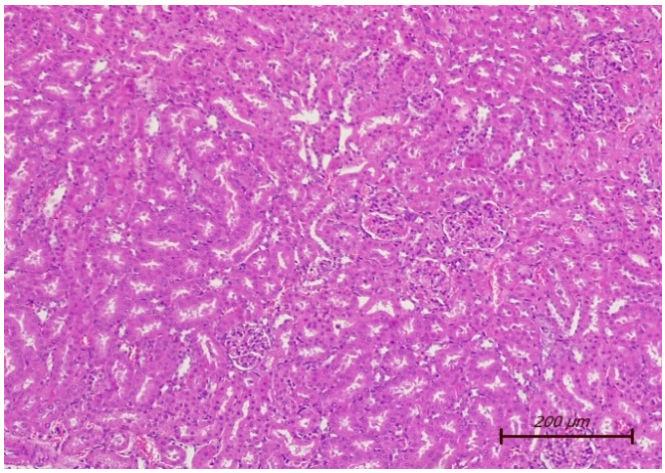
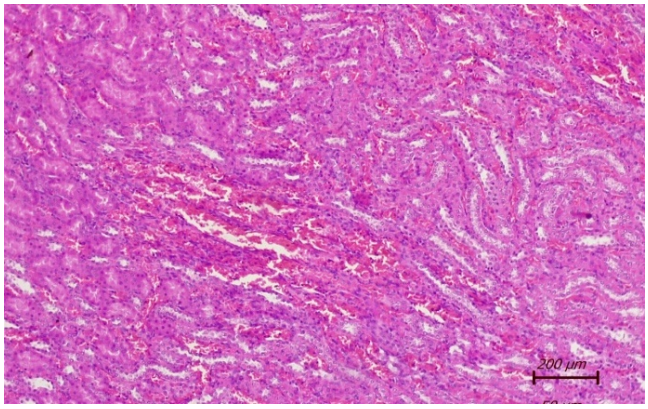
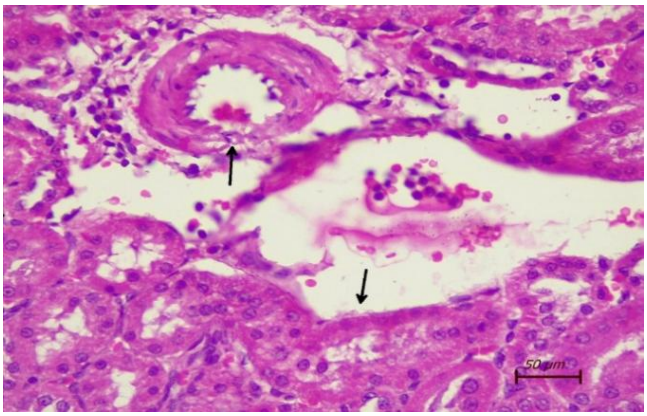


Figure 7: kidney section from rat (3 months) (control group) showing normal renal parenchyma, glomeruli, proximal convoluted tubules and distal convoluted tubules. (H&E, scale bar: 200 µm).

There is a correlation between various proinflammatory cytokines and insulin resistance/T2DM. In this study, pioglitazone decreased renal TNF-α and IL-1β. These results are similar to those of [38], who demonstrated that pioglitazone can reduce serum TNF-α levels by several mechanisms, including inhibition of TNF-α production from macrophages, suppression of TNF-α mRNA expression from subcutaneous adipose tissue, reduction of the number of CD3+ T lymphocytes in diabetic rats, producing higher levels of TNF-α and IL-1β and results of [39], who revealed that administration of pioglitazone effectively reduced TNF-α, IL-6, and IL-1β contents in the liver. Moreover, PPAR-γ agonists have been shown to inhibit expression of the TNF-α gene in the adipose tissue of obese rodents [40]. In the current study, the improvement in TNF-α and IL-β1 in rosuvastatin-treated diabetic rats was highly significant HFD/STZ control. The outcome of the influence of rosuvastatin on TNF-α in diabetic rats is in accordance with the findings of [26], who concluded that renal levels of TNF-α were significantly reduced in renal tissue when treated with rosuvastatin than diabetic untreated animals. It is known that statins have anti-inflammatory effects in both clinical practice and in experimental animal studies [41]. This is

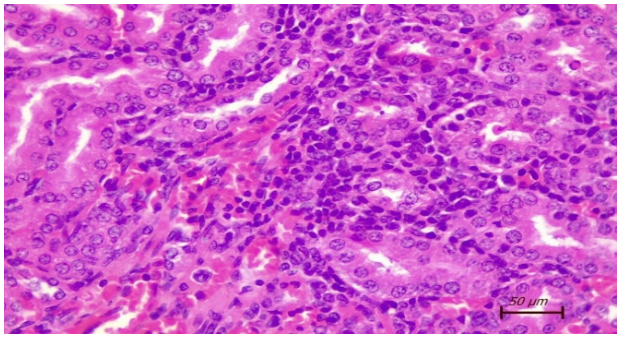


(a) kidney section from rat (3 months) (diabetic group) showing severe congestion and hemorrhage of renal parenchyma. (H&E, scale bar: 200 µm).

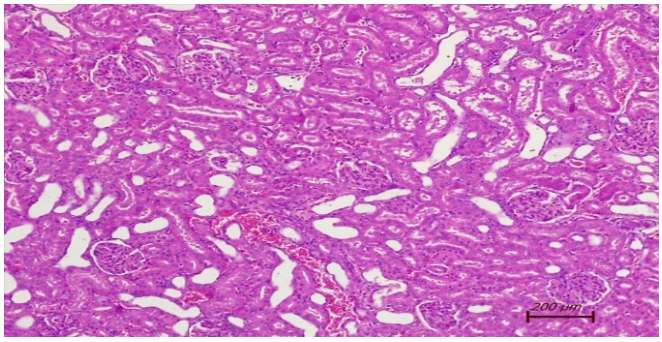


(b) kidney section from rat (3 months) (diabetic group) showing injury of intima of blood vessels (arrows). (H&E, scale bar: 50 µm).

Figure 8



(a) kidney section from rat (3 months) (diabetic group) showing hemorrhage of renal parenchyma and focal aggregation of lymphocytic cells in interstitial tissue. (H&E, scale bar: 50 µm).



(b) kidney section from rat (3 months) (diabetic group) showing congestion, and tubular atrophy in renal parenchyma. (H&E, scale bar: 200 µm)

Figure 9

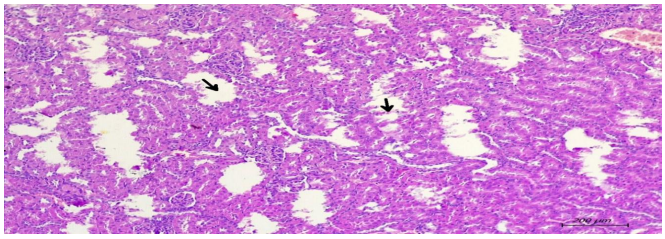


Figure 10: kidney section from rat (3 months).(Diabetic rats treated with pioglitazone) showing extensive degeneration and total loss of some renal tubules (black arrow) and some pycnotic nucleus. (H&E, scale bar: 200 µm)

in disagreement with [42], who observed that rosuvastatin treatment did not affect the elevated expression of TNF-α in the adipose tissue of HFD rats with respect to controls.

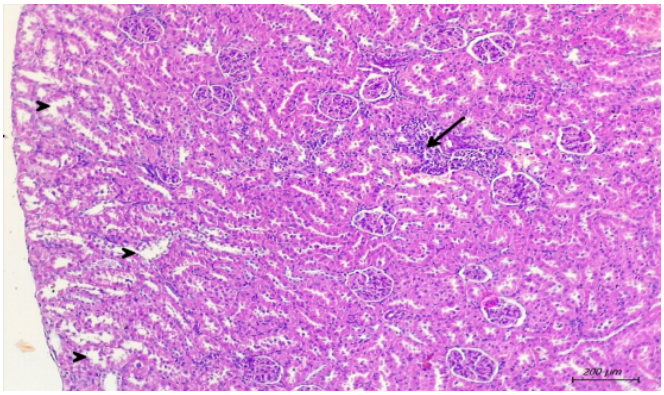


Figure 11: kidney section from rat (3 months).(Diabetic rats treated with rosuvastatin) showing focal interstitial lymphocytic cell infiltration (black arrow), some pycnotic nucleus and vacuolar degeneration of renal tubules in renal cortex (arrow heads). (H&E, scale bar: 200 µm).

Pioglitazone plus rosuvastatin reduced TNF-α and IL-1β levels more than pioglitazone or rosuvastatin alone in diabetic rats.

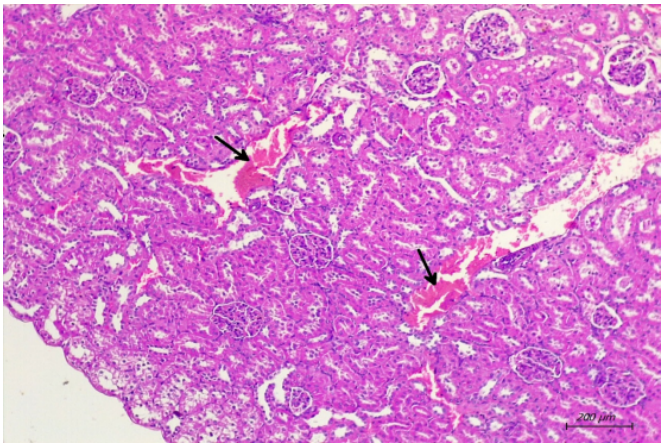


Figure 12: kidney section from rat (3 months).(Diabetic rats treated with rosuvastatin and pioglitazone) Showing vascular cortical/medullary hemorrhage/congestion (arrows). (H&E, scale bar: 200 μm).

Conclusion:

Our data suggested that the use of pioglitazone or rosuvastatin, either alone or combined, resulted in an improvement in DN through an anti-inflammatory mechanism in diabetic rats.

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