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RESEARCH ARTICLE

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Fenofibrate improves renal function by amelioration of NOX-4, IL-18, and p53 expression in an experimental model of diabetic nephropathy

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Abstract

Among several pathological mechanisms involved in diabetic nephropathy, oxidative stress, inflammation, and apoptosis play a prominent role. Fenofibrate, a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, has markedly improved oxidative stress and inflammatory responses, but there is no evidence about its effects on interleukin-18 (IL-18), NADPH oxidase type 4 (NOX-4), and p53 expression in diabetic kidneys. The aim of this study was to evaluate possible effects of fenofibrate on improving the underlying mechanisms of diabetic nephropathy. Male Wistar rats were randomly divided into four groups namely, normal, normal treated, diabetic and diabetic treated ($N = 6$). Diabetes was induced by a single dose of streptozotocin (40 mg/kg; IV). Treated animals received fenofibrate for 8 weeks daily (80 mg/kg; po). All groups were sacrificed on day 56 and blood, urine, and tissue samples were collected. Serum levels of urea, uric acid, creatinine, and glucose were assessed. Then, serum levels of malondialdehyde (MDA), nitrate, and glutathione (GLT), as well as the activities of catalase (CAT) and superoxide dismutase (SOD) enzymes were measured. The expression level of NOX-4, IL-18, and p53 proteins at both mRNA and protein levels were evaluated. Diabetes significantly increased albuminuria, free radicals production, inflammation, and apoptosis in non-treated rats while lowered antioxidant capacity. Moreover, diabetes caused histological damages leading to renal failure. Treatment with fenofibrate improved renal function by improving creatinine clearance ($P = 0.01$) and protein excretion ($P = 0.001$) and lowering plasma levels of blood urea nitrogen ($P = 0.001$), creatinine ($P = 0.001$), and uric acid ($P = 0.01$). Fenofibrate potentiated antioxidant defense systems by enhancing CAT ($P = 0.01$) and SOD ($P = 0.01$) enzymes activities and GLT content ($P = 0.01$), and reduced oxidative damage by lowering MDA generation ($P = 0.02$). Fenofibrate also attenuated the expression of NOX-4 ($P = 0.05$), IL-18 ($P = 0.05$), and p53 ($P = 0.05$) at both mRNA and protein levels. In conclusion, treatment with fenofibrate improved renal function by suppression of oxidative stress, attenuation of inflammation, and inhibition of apoptosis.

KEYWORDS
diabetic nephropathy, fenofibrate, IL-18, NOX-4, PPAR-α agonist, p53

1 | INTRODUCTION

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) worldwide.[1] Although There are many pathophysiological pathways involved in DN, for example, deregulations in the renin-angiotensin system (RAS), protein kinase C (PKC) activation, and polyol pathway, the roles of inflammation and oxidative stress are more predominant.[2] Recent evidence indicates that inflammation and oxidative stress have stimulatory effects on other molecular pathways involved in DN.[2]

It has been proven that antioxidant defense system (ADS) elements could be overwhelmed by free radicals overproduction during un-controlled diabetes consequently resulting in oxidative stress.[3] Oxidative stress has an upstream influence on other pathophysiological pathways and triggers their activation.[3] Oxidative stress plays a pivotal role in DN development through stimulation of PKC, RAS, TGF-β production, inflammatory responses, TNF-α secretion, etc.[3] Different mechanisms are involved in free radicals production during hyperglycemia, but activation NADPH oxidase (NOX) enzyme has a more pronounced effect compared to others.[4,5] NOX is a membrane-bound enzymatic complex which consists of six subunits by 7 different homologs, among which type 4 (NOX-4) has predominant expression in renal tissues especially in glomerulus, interstitial, and mesangial cells as well as podocytes.[5,6] In vitro studies have shown that hyperglycemia-induced oxidative stress in kidneys is NOX-4-dependent and NOX-4-derived free radicals are strongly involved in oxidative stress expansion.[6,7] Moreover, Vicki and colleagues have shown that *NOX-4* gene knockout in diabetic animals has reno-protective effects by normalizing PKC activation and vascular endothelial growth factor (VEGF) expression.[8]

Other underlying processes which are closely associated with the pathophysiology of DN are inflammatory responses.[9] It has been proven that various types of cytokines and inflammatory mediators are overproduced during un-controlled diabetes.[9] In this context, the role of interleukine-18 (IL-18) is of particular importance. In this regard, it was demonstrated that IL-18 is highly associated with the onset of renal injuries compared to other diabetic complications.[10] Moreover, Nakamura et al indicated that elevated urinary and serum levels of IL-18 in diabetic patients are closely correlated with albuminuria.[11] IL-18 is a potent immunoregulatory cytokine which induces interferon-γ (INF-γ) activation and then triggers a cascade of inflammatory responses leading to destructive processes and DN development.[10] IL-18 also stimulates ICAM-1, IL-1, and TNF-α up-regulation and induces glucose-mediated endothelial cells apoptosis.[11,12] Therefore, pharmacological interventions capable of modifying IL-18 expression may delay the onset of DN.[10]

It has been established that both oxidative stress and inflammatory responses exert their cytotoxic effects via inducing apoptosis particularly through activation of p53 protein.[13,14] High levels of oxidative stress can stimulate pro-oxidant aspects of p53 protein activity, induce more cytotoxic effects and apoptosis which are both linked to increased cell death.[15] Also, inflammatory cytokines can up-regulate p53 gene expression level and induce its cytotoxic impacts.[16] Thus, elevated p53 expression during diabetes is a main marker of cellular death and tissue damage.

Peroxisome proliferator activated receptor-alpha (PPAR-α) is a family of nuclear transcription factors which are highly expressed in tissues with oxidative capacity such as kidneys.[17] It has been recognized that these nuclear receptors have potent regulatory effects on lipids and carbohydrates metabolism particularly in tissues with high oxidative capacity (eg, kidneys) and are thus strongly involved in the maintenance of oxidative balance during hyperglycemia.[17] Also, it has been shown that the PPAR-α agonist, fenofibrate, can ameliorate oxidative stress by reducing NOX and suppressing inflammation in hypertensive rats.[18] However, there is no evidence on the renal effect of fenofibrate in diabetes. In the current study, we evaluated the effects of fenofibrate on NOX-4, IL-18, and p53 expression in DN.

2 | METHODS

2.1 | Animals

Male Wistar rats (190-220 g) were purchase from Pasteur institute, Tehran, Iran and randomly divided into four groups namely, normal (N), normal treated (FC), diabetic (D), diabetic treated (DF) ($N = 6$). All local and global ethical rules about experimental animals were considered and respected. Animals were kept in clean polyester cages in laboratory room under standard conditions (with 12-12 h light-dark cycles at 25°C).

2.2 | Drugs preparation

Streptozotocin (STZ) (Sigma-Aldrich) was dissolved in cold distilled water immediately before injection. Fenofibrate was purchased from Osveh Company (Iran) and daily prepared with CMC (carboxymethyl cellulose) as suspension (0.5%).

2.3 | Diabetes induction

Diabetes was induced by a single intravenous injection of STZ into tail vein (40 mg/kg). Then, 72 h after injection, blood glucose was determined by a glucometer (Bionime, Swiss) and animals with blood glucose higher than 400 mg/kg were considered as diabetic and randomly separated into two diabetic groups (diabetic and diabetic-treated).

2.4 | Tissue and blood sampling

Animals were sacrificed on day 56 and blood samples were directly collected from the heart. Blood samples were centrifuged (at 200*g* for 5-7 min) and then serum was separated for different blood tests. Moreover, urine samples were collected using metabolic cages during the last week of study.

Tissue samples were promptly collected after induction of deep anesthesia by ketamine (100 mg/kg) and xylazine (10 mg/kg) and sacrificing the rats on day 56. From each animal, one kidney was kept in formalin 10% for histological assessments. Also, for tissue samples preparation, 500 mg of tissue was added to a phosphate-buffered solution (pH 7) and then centrifuged (at 4000*g* for 45 min at 4°C). Then, supernatants were removed and kept at −70°C until used.

2.5 | Biochemical assessments

Blood tests involved determination of glucose, urea (BUN), creatinine, and uric acid in serum samples using standard commercial kits. Urine tests included determination of protein and creatinine excretion rate by standard kits. After assessment of creatinine concentration in both serum and urine samples and identifying 24-h urine volume, creatinine clearance was calculated using this formula:

$$\text{Creatinine clearance } (\text{mg/mL}/1.73\ \text{m}^3) = (\text{Serum creatinine} \times 1440)/((\text{urine creatinine}) \times (\text{daily urine volume}))$$

2.6 | Oxidative stress tests

2.6.1 | SOD enzyme activity assay

The SOD enzyme activity was calculated as described by Winterbourn.[19] This technique is based on SOD enzyme ability to inhibit the reduction of nitro blue tetrazolium by superoxide free radical. In this method, 0.067 M potassium phosphate buffer (pH 7.8) was added to 0.1 M EDTA (ethylenediaminetetraacetic acid) containing 0.3 mM sodium cyanide, 1.5 mM nitro blue tetrazolium, and 0.1 mL of previously prepared tissue sample. Then, 0.12 mM of riboflavin was added to each sample to activate the reaction and the mixture was incubated for 10 min. The absorbance of samples was recorded at 560 nm using a spectrophotometer. The amount of enzyme required to produce 50% inhibition was considered 1 unit (U) and the results were expressed as U/mL.

2.6.2 | CAT enzyme activity assessment

CAT enzyme activity was examined based on the method introduced by Aebi.[20] Here, 0.85 mL of potassium phosphate buffer 50 mM (pH 7.0) and 0.1 mL of the tissue homogenate was incubated for 10 min at room temperature. By addition of 0.05 mL H_2O_2 (30 mM prepared in potassium phosphate buffer 50 mM, pH 7.0) reaction was induced. Then, absorbance was recorded for 3 min at 240 nm. Finally, enzyme activity was expressed as U/mL (1 μmole H_2O_2).

2.6.3 | GLT content evaluation

GLT content in renal tissue was measured using the method of Tietz.[21] Tissue cells' proteins were precipitated by sulfosalicylic acid 5%, then the mixture was centrifuged at 4500*g* for 15 min and supernatant was separated. To measure GLT content in supernatants, 100 μL of supernatant was added to 810 μL of 0.3 mM Na_2HPO_4 plus 90 μL of DTNB (5,5′-dithiobis(2-nitrobenzoic acid) in 0.1% sodium citrate medium. Next, the DTNB absorbance was recorded at 412 nm for 5 min. A standard curve for GLT was plotted and the sensitivity of measurement was determined between 1 and 100 μM. The level of GLT was expressed as nMol/mL.

2.6.4 | MDA content assay

The amount of MDA (malondialdehyde) as a marker of oxidative stress-induced lipid peroxidation was evaluated as previously explained by Satoh et al.[22] In this method, 0.5 mL of tissue homogenate was added to 1.5 mL of trichloroacetic acid 10% and centrifuged at 4000*g* for 12 min. Then, to 1.5 mL of supernatant, 2 mL thiobarbituric acid 0.67% was added and the mixture was put in sealed tubes and placed in a hot water bath for about 30 min. Samples were allowed to cool at room temperature for 20 min. Subsequently, 2 mL N-butanol was added and the mixture was centrifuged at 2000*g* for 5 min. The resulting supernatant was separated and its absorbance was measured at 532 nm using a spectrophotometer. MDA content was determined by using 1,1,3,3-tetraethoxypropane as standard. MDA concentrations were reported as nMol/mL.

2.6.5 | Nitrate content assay

Nitrate content of the "cytosolic extract" as a marker of nitric oxide was evaluated by the Griess colorimetric assay.[23] Protein-free cytosolic extract was obtained by addition of 0.01 mL of zinc sulfate to 0.4 mL of tissue sample followed by centrifugation at 4000*g* for 20 min at 4°C. Next, 0.3 mL of supernatant was added to 0.3 mL of chloride vanadium and incubated in dark. Afterward, 0.15 mL of sulfanilamide (0.01%) and 0.15 mL of N-[1-naphthyl] ethylenediamine dihydrochloride (NED, 0.01%) were added and incubated at 37°C for 30 min. Finally, the absorbance of solution was determined at 540 nm. Nitrite content was assessed using a standard curve generated from

the absorbance of each sodium nitrate solution and the nitrate levels were expressed as nMol/mL.

2.7 | Histological assessments

For assessment of probable micro-structural changes in kidneys, histological evaluations were performed following Hematoxylin-Eosin (H&E) staining. Two weeks after tissues removal, the kidney was fixed, dehydrated (by 70%, 80%, 96%, 100%, and 100% ethanol) and cleared (by xylene). Afterward, kidney samples were paraffin embedded and coronal serial sections (5-μm thickness) were prepared for staining. The samples were twice treated with xylene solution times (each time for 15 min) for clearing. The histological features were observed by an expert pathologist using a light microscope (Nikon, Japan) connected to a digital camera (CMEX, Holland) for capturing the micro-graphs.

2.8 | Gene expression assays

For evaluation of genes expression, we applied reverse transcription polymerase chain reaction (RT-PCR) technique in three steps namely, RNA extraction, cDNA synthesis, and amplification. For this purpose, 100 mL of tissue was mixed with 1 mL of topazol solution and after 15 min, 200 μL chloroform was added to the mixture and incubated at room temperature for 10 min. Then, the mixture was centrifuged at 12 000*g* for 15 min at 4°C) and supernatant was removed. Next, 500 μL isopropanol was added and centrifuged at 12 000*g* for 15 min at 4°C. Then, supernatant was separated and 1 mL ethanol was added to it and centrifuged at 7500*g* for 8 min at 4°C. By adding 70 μL DEBS solution to microtubes and incubating at 55°C for 5 min, total RNA was extracted.

cDNA synthesis was performed as follows: 3 μL of RNA and 17 μL of distilled water were added to cDNA synthesis commercial kit. Then, cDNA was synthesized in 12 rounds of three steps by thermo-cycler (step 1: at 20°C for 30 s; step 2: at 45°C for 4 min; and step 3: at 55°C for 30 s) plus to one round of heat activation step (55°C for 5 min).

For cDNA amplification, 3 μL of cDNA, 2 μL of primers (forward and reverse, Table 1), and 17 μL of distilled water were added to commercial PCR kit and then inserted into thermal cycler for six heating steps at 35 cycles (step 1: at 95°C for 2 min; step 2: at 95°C for 30 s; step 3: at 53°C for 30 s; step 4: at 72°C for 1 min; step 5: at 72°C for 10 min; and step 6: at 30°C for 30 s).

For running amplified genes, we applied agarose gel and beta-actin was used as the house keeping gene. Then, gels were kept in ethidium bromide for 20 min and photos were taken in Gel-Doc.

2.9 | Protein expression assays

For exact detection of protein expression levels, we applied immunohistochemistry (IHC) method as follows. Tissue samples were washed four times with phosphate-buffered saline (PBS) with 5 min intervals. Samples were treated with paraformaldehyde 4% for 20 min and HCl 2N for 30 min; then, borate-buffered saline was added for neutralization. Tissues were washed again. Trition 3% was added for cells disruption and cells were washed again with PBS. Samples were incubated with goat serum 10% for 30 min, and diluted primary antibody (1/100 in PBS) for 12 h at 4°C. Next, tissues were washed with PBS 4 times. Then, diluted secondary antibody (1/200) was added and incubated at 37°C for 90 min and samples were moved to a dark room, washed with PBS four times and treated with DAPI solution. Finally, samples were observed by fluorescent microscope for imaging.

2.10 | Data analysis

For analyzing data, Kolmogorov-Smirnov test was applied for examining normal distribution of data. Then, one way ANOVA (for assessing differences among groups), paired sample *T* test (for assessing differences in group) and also Tukey's as post hoc were applied. Data are expressed as mean ± SD and a $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | Determination of the blood levels of glucose, uric acid, creatinine, and BUN

Changes in blood glucose concentration (mg/dL) in different group are depicted in Figure 1A. In normal animals, mean blood glucose on the first day was 103 ± 9 mg/dL which declined to 96 ± 18 mg/dL, however, this change was not significant. Diabetes induction significantly increased blood glucose to 453 ± 35 mg/dL ($P = 0.001$). Fenofibrate in both normal and diabetic groups had no significant effects.

Regarding blood levels of uric acid, in normal group, mean blood level of uric acid was initially 2.65 ± 0.36 mg/dL which declined to 2.58 ± 0.6, but this change was not significant. Diabetes induction significantly raised blood uric acid to 5.02 ± 0.37 mg/dL ($P = 0.001$). Though fenofibrate administration led to no significant effect in normal animals, it decreased the level of uric acid to 3.67 ± 0.69 mg/dL ($P = 0.01$) in diabetic group.

Moreover, in normal group, mean blood creatinine on the 1st and 56th days were 0.63 ± 0.17 and 0.65 ± 0.14 mg/dL, respectively. Diabetes significantly raised blood creatinine to 5.2 ± 0.4 mg/dL ($P = 0.001$). Fenofibrate had no significant effect in normal rats, but in diabetic animals, it reduced blood creatinine levels to 1.66 ± 0.23 mg/dL ($P = 0.001$).

Blood BUN levels (mg/dL) of all experimental animals are shown in Figure 1D. In normal group, mean BUN value on the 1st and 56th days were 52.3 ± 10 and 54.6 ± 7.2 mg/dL, respectively, which did not show significant differences.

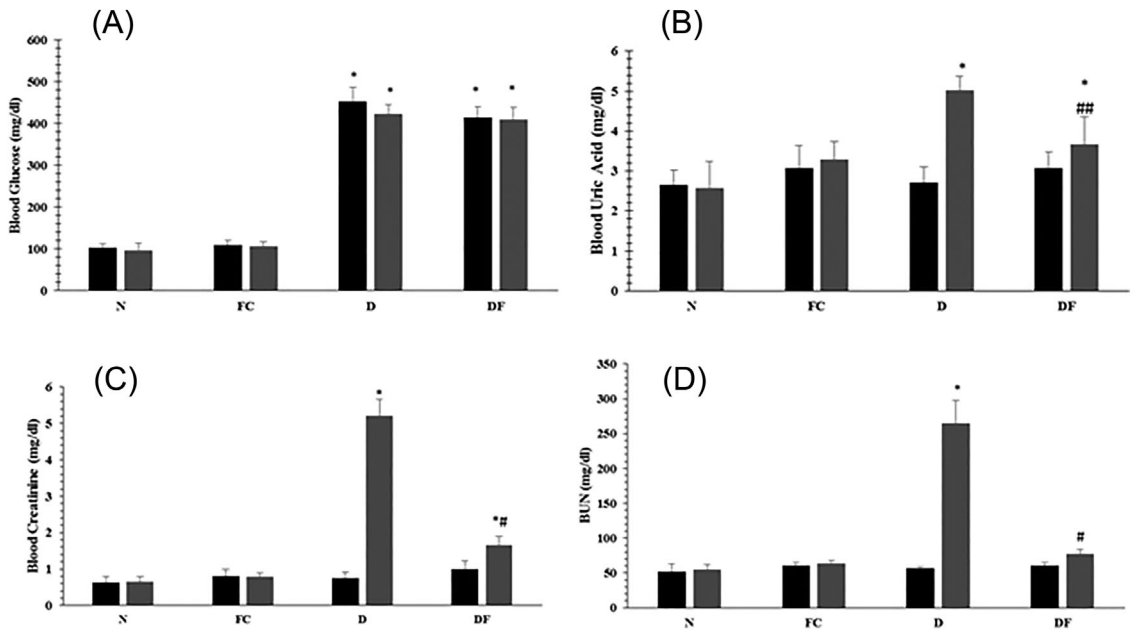

FIGURE 1 Blood levels of (A) glucose (mg/dL), (B) uric acid (g/dL), (C) creatinine (mg/dL), and (D) BUN (mg/dl) in normal (N), normal + Fenofibrate (FC), diabetic (D), and diabetic + Fenofibrate (DF) groups, on day 1 (the left bar at each point) and day 56 (the right bar at each point). All values are presented as Mean ± SD. A, $*P < 0.0001$ shows significant differences compared to the normal group (N) on the 1st and 56th day. B, $*P < 0.001$ shows significant differences compared to normal group (N) on the 56th day. $\#P < 0.01$ shows significant differences compared to diabetic group (D) on the 56th day. C, $*P = 0.001$ shows significant differences compared to normal group (N) on the 56th day. $\#P = 0.001$ shows significant differences compared to diabetic group (D) on the 56th day. D, $*P < 0.001$ shows significant differences compared to normal group (N) on the 56th day. $\#P < 0.00$ shows significant differences compared to diabetic group (D) on the 56th day

Diabetes significantly increased BUN to 265.25 ± 32 mg/dL ($P = 0.001$). Fenofibrate had no significant effect on BUN level in normal animals, but significantly declined BUN levels to 76.93 ± 6.6 mg/dL, in diabetic group ($P = 0.001$).

3.2 | Determination of the level of anti-oxidative stress components of the blood

The levels of CAT enzyme activity (U/mL) in different groups are illustrated in Figure 2A. In normal and normal treated animals, CAT activity levels were 48 ± 5.3 and 40 ± 6.5 U/mL, respectively, which exhibited no significant difference. Diabetes induction significantly declined CAT activity to 10.2 ± 1.34 U/mL ($P = 0.001$). Also, fenofibrate significantly increased CAT enzyme activity to 35.55 ± 5.03 U/mL ($P = 0.01$).

SOD enzyme activities (U/mL) in normal animals, was 0.65 ± 0.05 U/mL. Diabetes significantly declined this value to 0.23 ± 0.06 U/mL ($P = 0.001$). Fenofibrate increased SOD activity in normal (0.98±.06 U/mL) and diabetic (0.58 ± 0.08 U/mL) animals ($P = 0.01$).

Concerning GLT content, there were no significant differences between normal and normal treated groups (2810 ± 351 and 2731 ± 322 nmol/mL, respectively). GLT content in animals with uncontrolled diabetes was significantly reduced to 1452 ± 110 nmol/mL ($P = 0.001$). But, treatment with fenofibrate restored GLT content to near-normal levels (2496 ± 344 nmol/mL; $P = 0.01$) (Figure 2C).

3.3 | Determination of the levels of oxidative stress markers in the blood

According to our results, nitrate content (mmol/mL) in normal rats (28.65 ± 2.1 nmol/mL) was significantly different from that of normal treated animals (47.68 ± 3.2 nmol/mL) ($P = 0.01$). While diabetes significantly reduced this value to 18.65 ± 1.56 nmol/mL ($P = 0.01$), treatment with fenofibrate significantly improved it ($P = 0.04$; Figure 3A).

The changes in MDA content (as nMol/mL) are presented in Figure 3B. MDA levels in normal and normal treated animals were 8.62 ± 0.32 and 9.68 ± 0.7 nMol/mL, respectively, which were not significantly different. Diabetes significantly increased this value to 14.25 ± 0.23 nMol/mL ($P = 0.001$). But treatment with Fenofibrate significantly decreased this value to 10.18 ± 0.56 nMol/mL ($P = 0.02$).

Representative changes of proteinuria (mg/day) are presented in Figure 4A. The levels of proteinuria in normal and normal treated animals were 4.6 ± 0.82 and 3.28 ± 0.55 mg/day, respectively, which were not significantly different. Diabetes significantly increased proteinuria

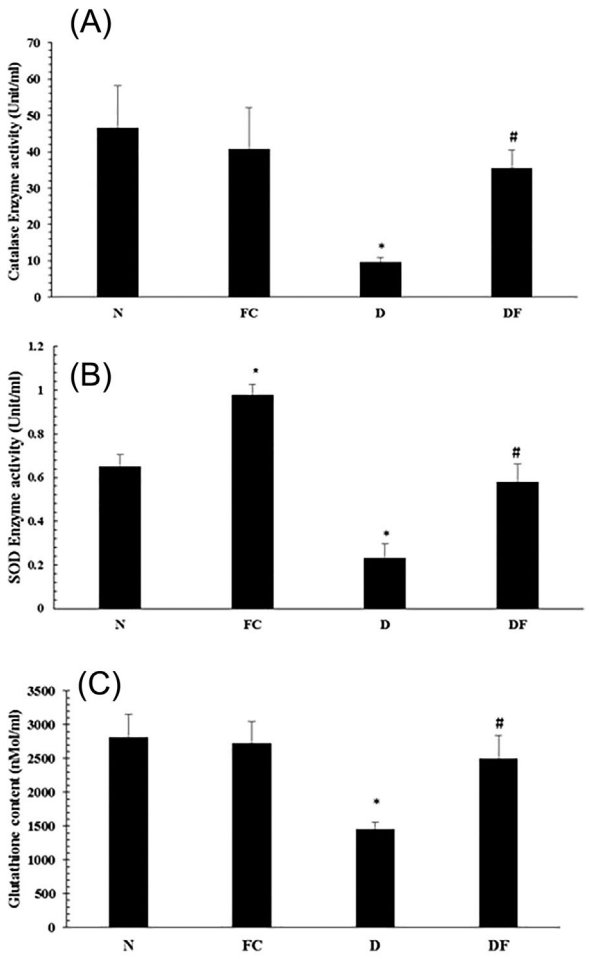

FIGURE 2 Representative changes of (A) Catalase Enzyme activity (U/mL) (B) SOD Enzyme activity (as U/mL) and (C) Glutathione content (nmol/mL) in normal (N), normal + Fenofibrate (FC), diabetic (D), and diabetic + Fenofibrate (DF) groups. All values are presented as Mean ± SD. A, $*P < 0.00$ shows significant differences compared to normal group (N). $\#P < 0.01$ shows significant differences compared to diabetic group (D). B, $*P < 0.01$ shows significant differences compared to normal group (N). $\#P < 0.01$ shows significant differences compared to diabetic group (D). C, $*P < 0.00$ shows significant differences compared to normal group (N). $\#P < 0.01$ shows significant differences compared to diabetic group (D)

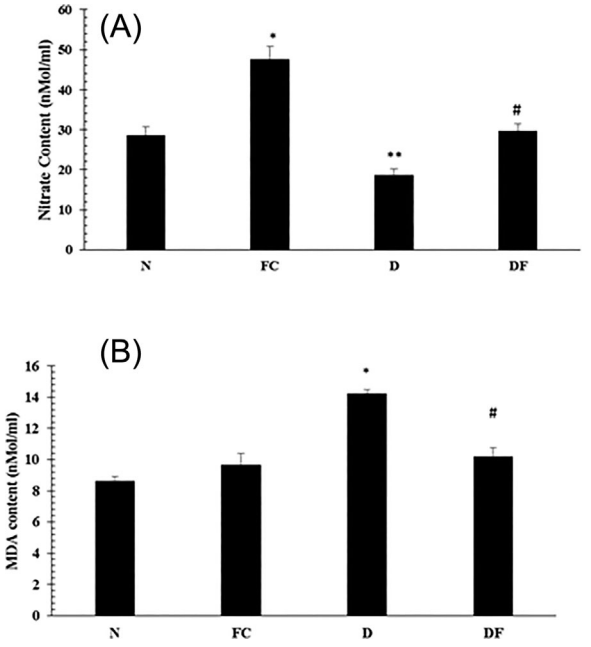

FIGURE 3 Representative changes in (A) Nitrate content (as nMol/ml) and (B) MDA content (nMol/ml) in normal (N), normal + Fenofibrate (FC), diabetic (D), and diabetic + Fenofibrate (DF) groups. All values are presented as Mean ± SD. A, $*P < 0.01$ and $**P = 0.01$ show significant differences compared to normal group (N). $\#P = 0.04$ shows significant differences compared to diabetic group (D). B, $*P < 0.00$ shows significant differences compared to normal group (N). $\#P < 0.02$ shows significant differences compared to diabetic group (D)

up to 63.2 ± 5.36 mg/day ($P = 0.001$), but Fenofibrate declined it to 29.07 ± 2.1 mg/day ($P = 0.001$), although it was yet greater than that of the normal group ($P = 0.001$).

Figure 4B presents changes in urinary creatinine excretion (mg/day) in all experimental animals. In normal and normal-treated groups, urinary creatinine excretion were 20.75 ± 2.55 and 23.86 ± 4.65 mg/day, respectively, which were not significant different. Diabetes declined this value to 5.59 ± 0.38 mg/day ($P = 0.001$). Treatment with Fenofibrate in diabetic group increased this value to 13.6 ± 0.83 mg/day ($P = 0.001$), but was much lower than that of the normal animals ($P = 0.01$).

Changes in the creatinine clearance (mg/min/1.73 m^2) are represented in Figure 4C. Mean value of creatinine clearance in normal and normal treated animals were 0.35 ± 0.03 and 0.3 ± 0.05 mg/min/1.73 m^2 which were not significantly different. Uncontrolled diabetes significantly declined this value to 0.037 ± 0.008 mg/min/1.73 m^2 ($P = 0.001$). Treatment with Fenofibrate in diabetic animals increased this value to 0.15 ± 0.02 mg/min/1.73 m^2 ($P = 0.01$), although it was lower than that of normal animals ($P = 0.001$).

3.4 | Effects of fenofibrate on the expression levels of IL-18, NOX-4, and p53

Fenofibrate exerted no significant effect on IL-18, NOX-4, and p53 expression. Diabetes increased the expression levels of the foregoing genes ($P = 0.01$), but fenofibrate declined these levels in diabetic rats ($P = 0.05$) (Figure 5). These findings at the gene expression level were corroborated at the protein level using IHC (Figure 6).

3.5 | Histopathological studies of renal tissues

As shown in Figure 7, glomerular sclerosis and expansion of mesangial space as two major criteria of renal lesions in DN,

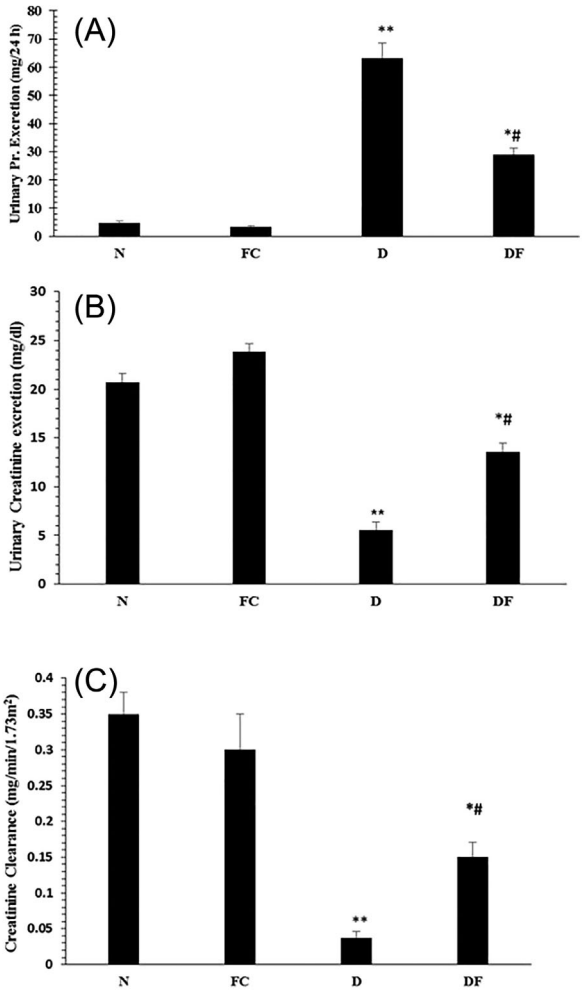

FIGURE 4 Representative changes of (A) Proteinuria (mg/day), (B) Urinary Creatinine Excretion (mg/day), and (C) Creatinine clearance (mg/min/1.73 m^2) in normal (N), normal + Fenofibrate (FC), diabetic (D), and diabetic + Fenofibrate (DF) groups. All values are presented as Mean ± SD. A, $^{**}P < 0.000$ and $^{*}P < 0.00$ show significant differences compared to normal group (N). $^{\#}P < 0.00$ shows significant differences compared to diabetic group (D). B, $^{**}P < 0.000$ and $^{*}P < 0.01$ show significant differences compared to normal group (N). $^{\#}P < 0.00$ shows significant differences compared to diabetic group (D). C, $^{**}P < 0.000$ and $^{*}P < 0.00$ show significant differences compared to normal group (N). $^{\#}P < 0.01$ shows significant differences compared to diabetic group (D)

were clearly seen in diabetic group, but fenofibrate improved this damage in diabetic animals.

4 | DISCUSSION

The current study investigated the role of inflammation, oxidative stress, and apoptosis in DN development and the counterbalancing potential of fenofibrate as a PPAR-α agonist, to ameliorate the above-mentioned pathophysiological mechanisms. Our results demonstrated that uncontrolled hyperglycemia reduced uric acid, urea and creatinine excretion, and deteriorated renal function. Also, uncontrolled hyperglycemia reduced nitric oxide levels which may lead to hemodynamic changes. We found that diabetes suppresses CAT, SOD and GLT activity, mitigates ADS function and enhances oxidative stress by increasing MDA production in renal tissue. Enhanced *NOX-4* expression has been suggested as a main reason for free radicals overproduction in diabetic kidneys. Our findings showed that diabetes induces IL-18 and p53 protein expression in kidneys which may result in increased rates of apoptosis. Treatment with fenofibrate lowered oxidative stress, improved inflammation, and decreased *p53* expression level in diabetic animals. Together, we found that fenofibrate protects the kidney against diabetes-induced damages. This finding is consistent with previous studies suggesting pleiotropic effects of fibrates besides their putative triglyceride-lowering properties.[24–26] Fibrates are known to possess antioxidant effects[27,28] and also ameliorate some glycemic[29] and thrombotic[30–32] factors, reduce uric acid,[33] increase adiponectin,[34] and enhance endothelial function.[35]

Oxidative stress has an important role in various organs' failure including renal failure.[3,8] During diabetes, free radicals are over-produced by several pathways, particularly through NOX enzyme hyperactivity.[5,6,22] Previous studies have shown that in kidneys, NOX-4 isoform of this enzyme is expressed at higher levels compared to other isoforms.[5,8] In our study, *NOX-4* gene expressions in diabetic rats were elevated. Different studies have demonstrated that diabetes upregulates NOX-4 enzyme.[4,8] NOX-4 enzyme-induced free radicals overproduction has several deleterious effects on renal function.[5] These free radicals can directly attack cellular proteins and break them or oxidize lipid membranes and produce harmful by-products such as MDA.[22] In the present study, we showed that NOX-4 enzyme is upregulated in diabetes at both gene and protein levels.

NOX-4-induced free radicals overproductions in kidneys are closely associated with endothelial cells dysfunction, extracellular matrix expansion, inflammatory responses and cell proliferation, fibrosis and angiogenesis processes, and vascular remodeling leading to hemodynamic changes.[36] Therefore, inhibition of oxidative stress can play a beneficial role in DN management. For this reason, we used fenofibrate, a PPAR-α agonist to improve ADS and suppress NOX-4 activity. In our study, fenofibrate treatment increased SOD, CAT, and GLT activity and reduced MDA production in renal cells. Also, fenofibrate reduced NOX-4 expression at both gene and protein levels. These findings showed that fenofibrate can suppress oxidative stress by potentiation of ADS elements. Consistent with these findings, fenofibrate increased BUN, uric acid, and creatinine excretion while significantly reduced proteinuria in diabetic animals to near-normal values.

TABLE 1 Forward and reverse primers used for RT-PCR

Gene	Forward primer	Reverse primer
NOX-4	AGATGTTGGGCCTAGGATTGTG	AGCAGCAGCAGCATGTAGAAGA
IL-18	CGCAGTAATACGGAGCATAAATGAC	GTAGACATCCTTCCATCCTTCACAG
p53	TTCCGAGAGCTGAATGAGGCCTTG	ACTGACCGGATAGGATTTCGCTGG

There is little evidence about the effect of PPAR-α agonists on NOX-4 enzyme expression in DN.[18] Hou et al suggested that activation of PPAR-α receptors inhibits oxidative stress by down-regulation of NOX enzyme and increasing SOD enzyme activity in hypertensive rats.[18] Also, Cheng and colleagues reported that PPAR-α ablation increases NOX-4 expression and treatment with fenofibrate reduced oxidative stress and albuminuria in diabetic animals.[37] Down-regulation of NOX-4 enzyme in the current study was associated with increased renal creatinine, uric acid and urea excretion and reduced albuminuria, suggesting that fenofibrate restores oxidative balance in diabetic kidneys by enhancing ADS and suppressing free radicals production and oxidative stress.

Inflammation is another mechanism involved in DN.[12] There is compelling evidence showing that circulatory

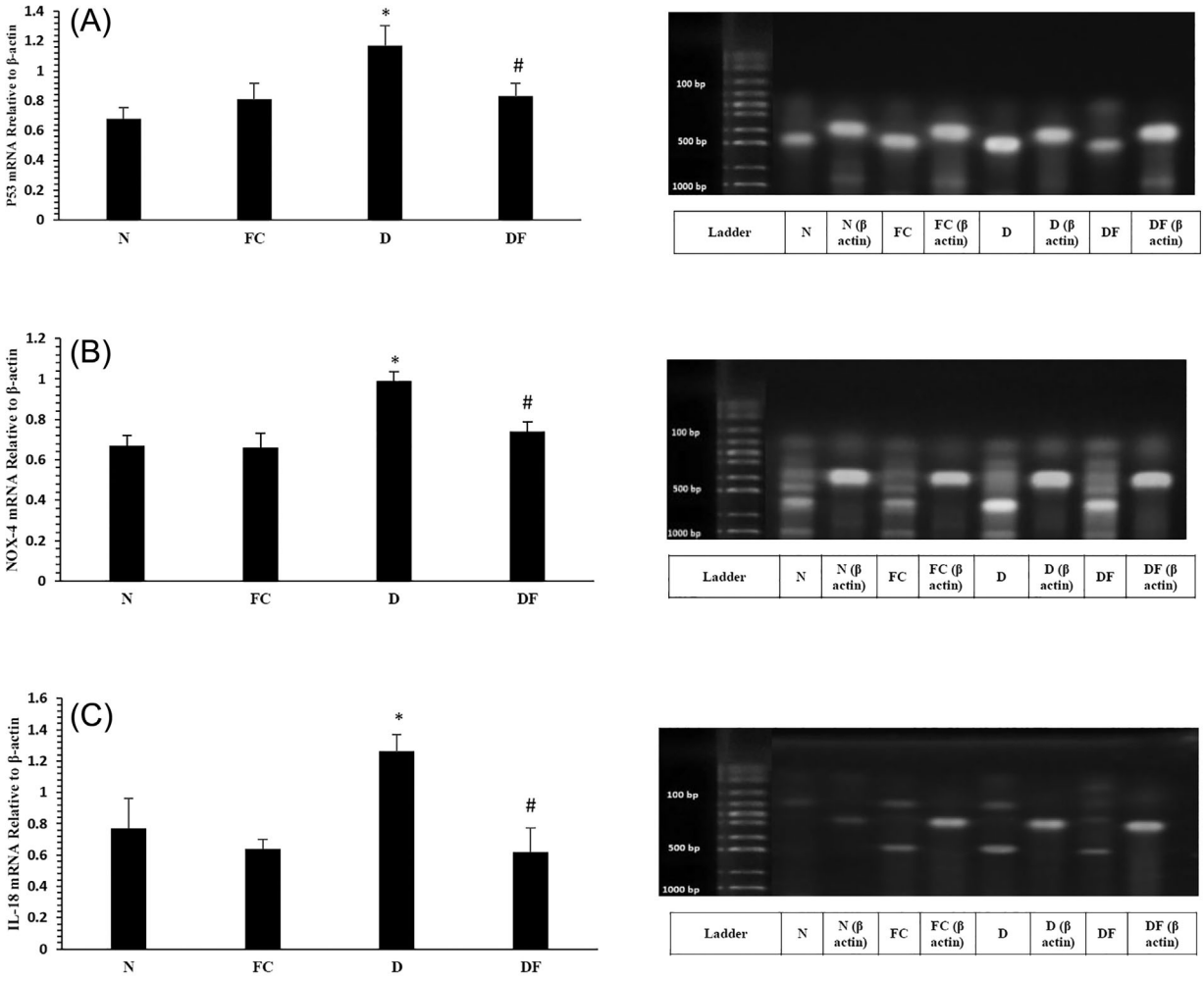

FIGURE 5 (A) IL-18, (B) NOX-4, (C) p53 expression level against β-actin in normal (N), normal treated (FC), diabetic (D), and diabetic treated (DF) groups. All values are presented as Mean ± SD. A, $P = 0.01$ shows significant differences compared to normal control group (N). $^{\#}P = 0.05$ shows significant differences compared to diabetic group (D). B, $^{*}P = 0.01$ shows significant differences compared to normal group (N). $^{\#}P = 0.05$ shows significant differences compared to diabetic group (D). C, $^{*}P = 0.01$ shows significant differences compared to normal group (N). $^{\#}P = 0.05$ shows significant differences compared to diabetic group (D)

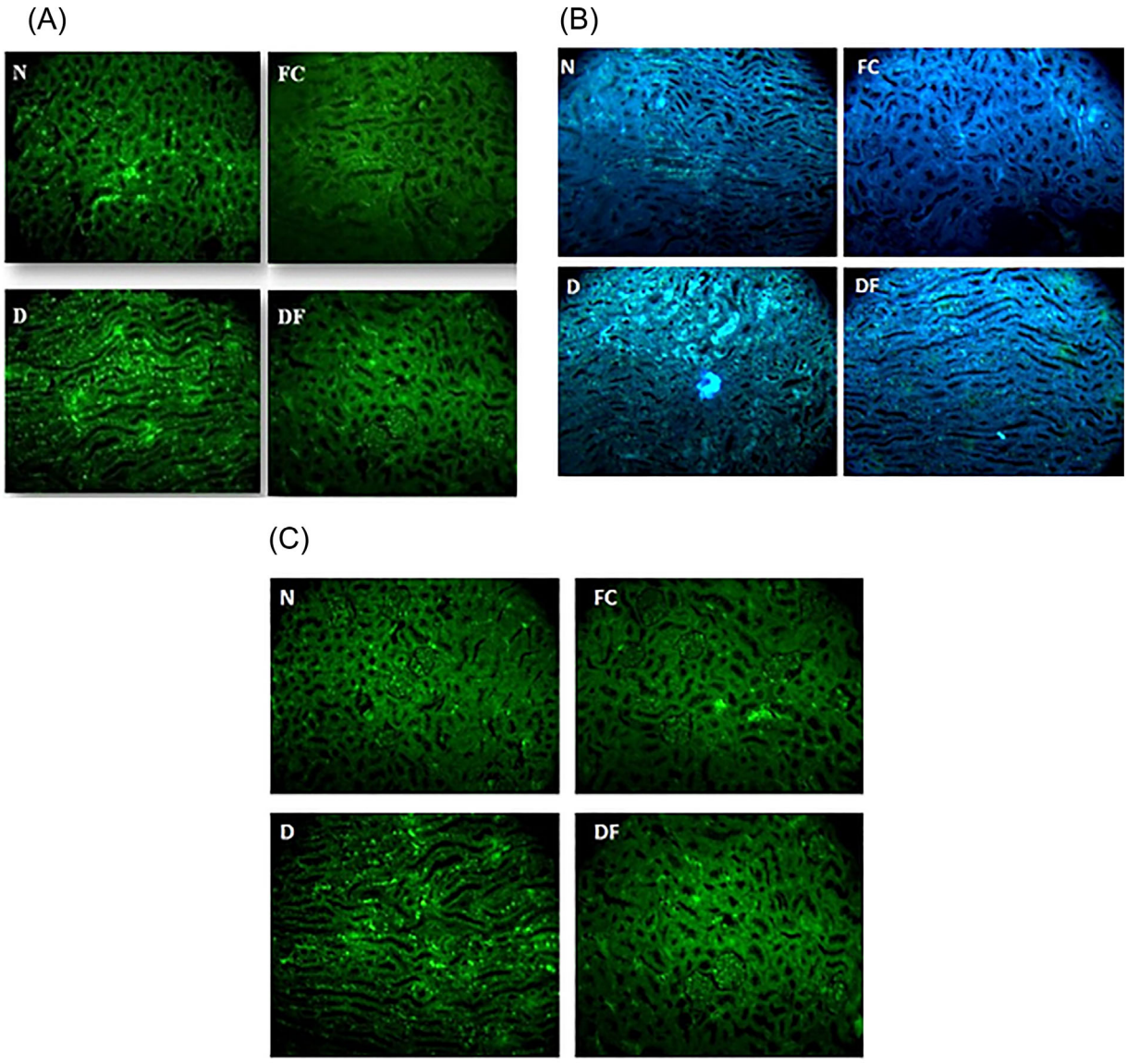

FIGURE 6 IHC images of (A) p53, (B) NOX-4, and (C) IL-18 protein production in normal (N), normal treated with fenofibrate (FC), diabetic (D), and diabetic treated with fenofibrate (DF) groups

inflammatory cytokines, leucocytes, monocytes, and adhesion molecules are closely implicated in developing DN.[2,12,38] Also, in vivo studies have demonstrated that immunosuppressive agents which suppress macrophage accumulation, reduce the risk of DN development.[39,40] In the current study, we evaluated the role of IL-18, as a potent pro-inflammatory mediator, in DN.[38,41] Previously, Fujita et al demonstrated that IL-18 association with DN is more marked than its effect on other diabetic complications.[10] Our results indicated that diabetes markedly increased both IL-18 gene and protein expressions. This is consistent with previous reports showing that hyperglycemia increases IL-18 either directly,[42,43] or indirectly via increasing free radicals.[38] In our study, enhanced IL-18 expression in diabetic rats was accompanied by histological damage and proteinuria, confirming that inflammation has a key role in renal failure in diabetic subjects. It has been illustrated that there is a direct association between urinary IL-18 level and albuminuria in diabetic patients.[42] Fenofibrate down-regulated IL-18 and reduced inflammation in the diabetic group. This effect was accompanied by increased creatinine, uric acid and urea excretion, lower histological damage, and improved renal function. To our knowledge, there has been no evidence about the effect of PPAR-α activation on IL-18 expression. Thus, our study, for the first time, showed that fenofibrate can down-regulate IL-18 and improve inflammation in diabetic milieu. It is suggested that fenofibrate inhibits IL-18 either directly through suppression of gene expression, as already shown for IL-17,[44] or indirectly via attenuation of oxidative stress.[18,45]

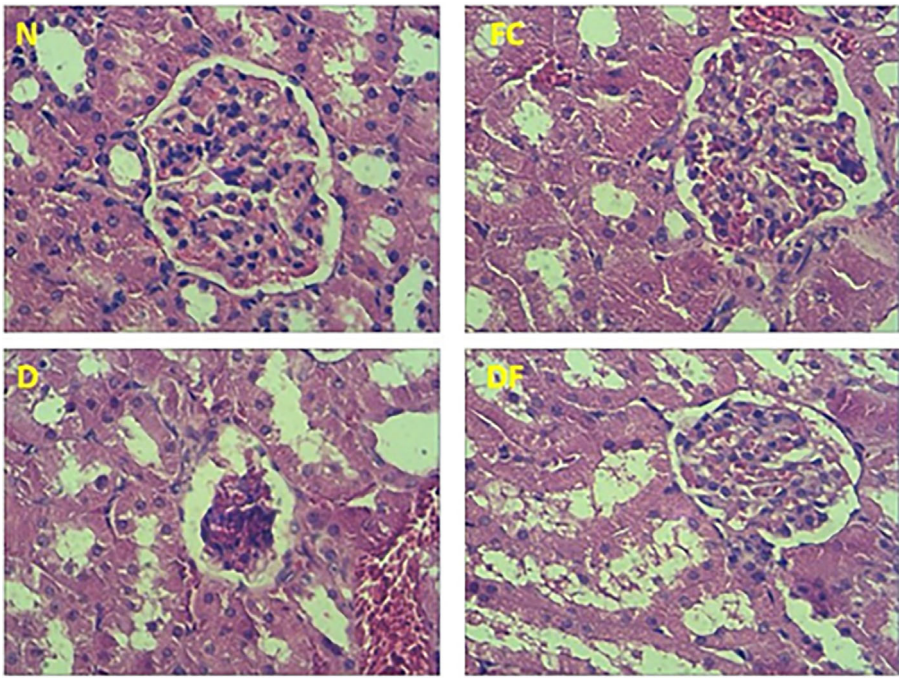

FIGURE 7 Histopathological studies of renal tissue in normal (N), normal treated (FC), diabetic (D), and diabetic treated (DF) groups

Hyperglycemia-induced apoptosis is a common phenomenon which may occur through Bax/Bcl-2 ratio modulation, p53 protein activation, etc.[46] Also, it is stated that both inflammation and oxidative stress may exert some of their deleterious effects through apoptosis induction via inducing p53 protein activity.[47,48] Therefore, apoptosis is involved in tubular, vascular, and glomerular injuries of DN.[49,50] In our study, diabetes markedly increased p53 expression at both mRNA and protein levels while fenofibrate treatment decreased p53 expression. There is little evidence about the effect of fenofibrate on p53 protein levels in diabetic milieu. Zanetti and coworkers showed that PPAR-α activation has an inhibitory effect on apoptosis in diabetic milieu.[46] Also, Tomizawa et al reported that fenofibrate reduces apoptosis and inflammation through adenosine monophosphate-activated protein kinase (AMPK) activation.[51] De Silva et al also demonstrated that pretreatment with fenofibrate reduces aldosterone-induced p53 up-regulation.[52] Taken all these evidence together, it can be suggested that the ameliorative effect of fenofibrate on renal function is, at least in part, mediated through reduction of apoptosis.

5 | CONCLUSION

Our findings indicated that oxidative stress, inflammatory responses (induced by IL-18 cytokine) and apoptosis are involved in the pathophysiology of DN. Un-controlled hyperglycemia can weaken ADS elements and enhance free radicals production resulting in oxidative stress; it can also induce inflammatory responses and IL-18 has a key role in this. Moreover, hyperglycemia induces p53 expression and apoptosis. These three pathways lead to albuminuria and renal failure. Our findings demonstrated that PPAR-α activation with fenofibrate can improve renal function in diabetic milieu via at least three mechanisms: (1) potentiation of cellular ADS, reduction of NOX-4 expression and suppression of oxidative stress; (2) lowering IL-18 cytokine expression and attenuation of inflammation; and (3) inhibition of p53 expression and p53-dependent apoptosis. Future studies are warranted to confirm these findings in clinical settings and also to explore if such effects are also exerted by the newly introduced selective PPAR modulators.[53,54]

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CONFLICTS OF INTEREST

The authors clearly declare that they have no conflicts of interest in this study.

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