Original Article

Renoprotective Potential Of Ranolazine In Ameliorating Diabetic Nephropathy In A Rat Model Of Streptozotocin-Induced Diabetes

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Abstract

Background: Diabetic nephropathy is one of the major consequences of diabetes mellitus and is defined as the progressive development of renal insufficiency in the setting of hyperglycaemia. Poor glycaemic control, inflammation, and oxidative stress are the primary mechanisms behind the pathogenesis of diabetic nephropathy in diabetic patients. Ranolazine is an anti-anginal drug shown anti-diabetic and anti-inflammatory effects in few preclinical and clinical trials in diabetes and coronary artery diseases. In the present study, we aimed to investigate the renoprotecive efficacy of ranolazine in ameliorating diabetic nephropathy in a rat model of streptozotocin induced diabetes

Methods: Male Wistar albino rats weighing 200±20 grams were used for our study. They were divided randomly into four groups of eight. Diabetic nephropathy was induced in three groups by injecting a single dose of 45 mg/kg streptozotocin. Distilled water (normal control), metformin 180mg/kg (metformin-treated group), and ranolazine 45mg/kg (Ranolazine treated group) were administered orally for 8 weeks and diabetic rats in the diabetic control group remained untreated. After the administration of ranolazine, weekly random blood glucose (RBS), HbA1c%, serum creatinine level, and urine albumin were examined. At the end of the experiment, rats were euthanized, and the serum was analyzed for TNF-α, IL-6, and CRP levels, and kidney sections were analyzed using hematoxylin and eosin (H & E) and PAS stain. Induction of diabetic nephropathy was confirmed by histopathological examination of the kidney.

Results: Ranolazine monotherapy significantly reduced random blood glucose($p<0.0001$), HbA1c%, urine albumin, serum creatinine, and inflammatory markers like CRP, IL-6, and TNF-α ($p\leq0.001$) as compared to the diabetic control group. Histopathological examination of the rat kidneys showed ranolazine was successful in preventing the changes in diabetic nephropathy such as basement membrane thickening, mesangial matrix expansion, interstitial nephritis, and focal tubular necrosis as compared to the diabetic control rats.

Conclusions: Ranolazine demonstrated renoprotection by significantly ameliorating diabetic nephropathy in a chronic model of streptozotocin-induced diabetes and needs further evaluation for its use in diabetic nephropathy.

Keywords: Ranolazine, Diabetes, Diabetic Nephropathy, Histopathology, Kidney disease STZ-induced diabetes

INTRODUCTION

Diabetic nephropathy (DNP) is a microvascular complication of type 1 and type 2 diabetes and is one of the major causes of renal failure.[1] More than 40% of diabetes patients develop chronic kidney disease, and most of them require renal replacement therapy because of renal failure.[2] DNP is characterized by albuminuria (proteinuria), hyperfiltration, hyperpermeability to macromolecules, and end-stage renal failure.[3] The kidney damage caused by DNP is histologically indicated by the thickening of the glomerular basement membrane, mesangial matrix expansion, macrophage infiltration, podocyte loss, and tubular epithelial degeneration.[4] DNP progression and tissue damage are exacerbated by poor glucose control, advanced glycation end products (AGEs), genetic predisposition, renin-angiotensin system activation, reactive oxygen species (ROS), and oxidative stress.[5] In the early stages of DNP, there seem to be no symptoms or indicators.[6] As the disease progresses, changes in blood pressure, fluid balance, and increased excretion of urine albumin are observed, and chronically raised blood glucose levels and inflammatory indicators affect the kidney's ability to filter blood.[6] In response to chronic hyperglycemia and AGE (Advanced Glycation End Products) accumulation, the renal parenchyma secretes monocyte chemoattractant protein (MCP-1) that attracts other immune cells to the kidneys. Monocytes activate and differentiate MCP-1 and start releasing inflammatory cytokines and reactive oxygen species (ROS).[7] Pro-inflammatory cytokines such as interleukin-1, interleukin-6, and interleukin-8, as well as CRP and ROS, cause renal parenchymal cell death and necrosis, as shown in Figure 1.[8] Profibrotic growth factors such as transforming growth factor and vascular endothelial growth factor (VEGF) encourage the formation of myofibroblasts, leading to fibroblast

proliferation and extracellular matrix deposition.[8] Currently, ACE inhibitors and ARB-1 are proven valuable in the management of DNP. These medications, on the other hand, are ineffective in preventing diabetic nephropathy.[9] Many drugs have demonstrated therapeutic benefits in animal research but have failed in clinical trials because of a lack of efficacy or safety concerns. There is a significant unmet demand in this therapeutic area. As a result, it was of interest to investigate novel agents for diabetic nephropathy therapy.[10]

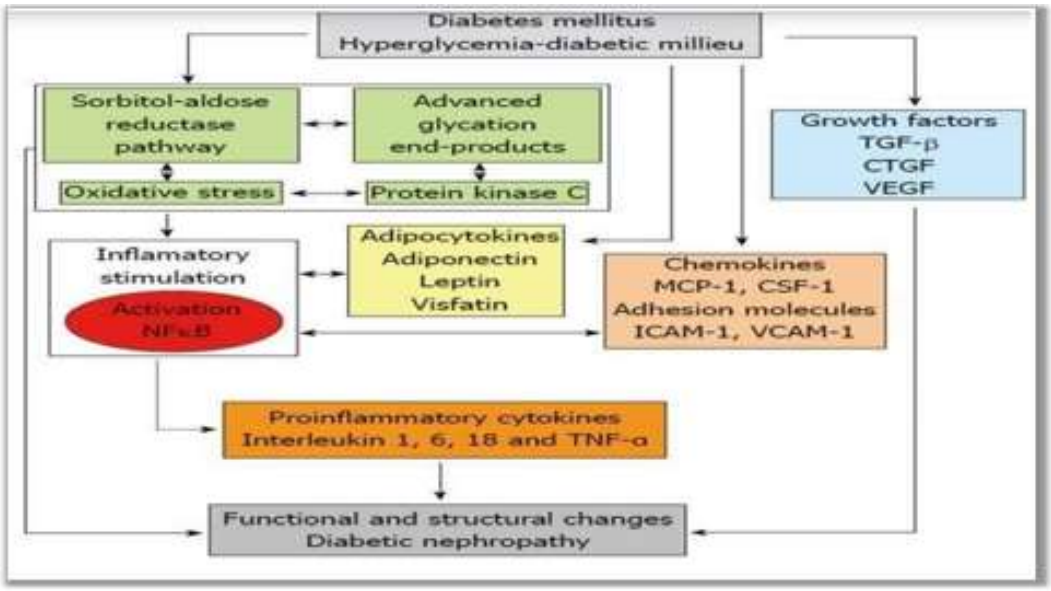

Fig.1: Pathophysiology of Diabetic nephropathy

Ranolazine is an anti-ischemic and antianginal drug prescribed for chronic angina treatment along with other antianginal medications.[11] Clinical trials have proven ranolazine to have positive metabolic benefits in diabetic people, as evidenced by considerable reductions in HbA1c levels.[12] The anti-diabetic action of ranolazine is due to the blockade of specific NaCh in alpha cells of the pancreas, which has glucagon- and glucose-lowering effects in animal models of diabetes.[13] Few in vivo studies have demonstrated the anti-inflammatory efficacy of ranolazine in various animal models.[14,15] Evidence suggests that the anti-diabetic and anti-inflammatory effects of ranolazine can help with the consequences of post-diabetic complications.[16,17]

Pharmacological treatment for diabetic nephropathy now relies on medications that affect the renin-angiotensin system. Despite the availability of many medications, there is a need for the development of drugs that can slow down the progression of the disease. Considering the disease's burden and the significance of inflammation in the pathogenesis of the disease, anti-diabetic drugs with additional anti-inflammatory actions can be investigated since they would benefit patients with comorbid conditions. Pre-clinical investigations demonstrated that ranolazine, an antianginal medication, has antihyperglycemic and anti-inflammatory characteristics. However, there is no evidence of animal studies that have tested the anti-diabetic and anti-inflammatory efficacy of ranolazine in preventing diabetic nephropathy. The present study investigates the Renoprotective efficacy of ranolazine in ameliorating diabetic nephropathy in a rat model of streptozotocin-induced diabetes via estimation of blood and urine glucose, inflammatory markers, renal functions, and renal histopathological examinations.

MATERIAL AND METHODS

Experimental Animals

The study was carried out on thirty-two male Wistar rats weighing about (200±20 grams) and aged around (12±2 week's age). They were obtained from the central animal house of Jawaharlal Nehru Medical College, Belagavi, Karnataka, India. The rats were randomly divided into four groups (n=8) and housed in polypropylene cages at an ambient temperature of 25±1ºC and 45-55% relative humidity. They were acclimated to a 12:12-h light/dark cycle for ten days before the day of the experiment, confirmed by the Institutional Animal Care and Use Committee (627/PO/Re/02/CPCSEA).

Induction of Experimental Diabetes

Diabetic Nephropathy was induced in three groups by injecting a single dose (45 mg/kg) of streptozotocin, excluding the normal control group.[18] A freshly prepared solution of STZ (45 mg/kg body weight) in 0.1 ml citrate buffer with pH 4.5 was injected intraperitoneally of volume 1 ml/kg body weight into overnight fasted rats. STZ-treated rats were given 5% glucose instead of water for 24 hours after diabetes induction to prevent hypoglycemic shock-related mortality.[19] After 72 hours of STZ administration, the rats with random blood glucose levels higher than 250 mg/dl were selected for the experiment. Wistar rats with random blood sugar below 250 mg/dl were excluded from the study. Following the confirmation of the diabetic state, the rats in each group were treated for eight weeks according to one of the treatment protocols.[20]

Experiment Design

The rats were divided into four groups and given the following drugs for eight weeks.

- Group 1 (normal control group): Normal rats received distilled water of 20 ml/kg body weight orally for eight weeks.
- Group 2 (diabetic control group): Diabetic rats received a single dose of intraperitoneal injection of STZ (45 mg/kg).
- Group 3 (diabetic rats treated with metformin): Diabetic rats received a single dose of intraperitoneal injection of STZ (45 mg/kg) and metformin (180 mg/kg/day) orally for eight weeks.
- Group 4 (diabetic rats treated with ranolazine): Diabetic rats received a single dose of intraperitoneal injection of STZ (45 mg/kg) and ranolazine (90 mg/kg/day) orally for eight weeks.

Sample Collection and Preparation

At the end of the study, all groups were anesthetized using sodium pentobarbitone (30 mg/kg), and blood samples were collected for serum preparation through cardiac puncture. After the blood collection, the rats were euthanized using a high dose of sodium pentobarbitone (90 mg/kg). Their kidneys were preserved for histopathological studies using hematoxylin and eosin staining.

Determination of Body Weight

The rats' body weights were recorded on days 0, 3, 15, 30, 45, and 56.

Determination of Blood and Urine Glucose

The blood samples were collected from the rats' tail veins, and random blood glucose levels were recorded using a glucometer on days 0, 3, 15, 30, 45, and 56. HbA1c % was assessed using a chemical analyzer on days 0 and 56. The urine was collected at the end of the study to measure urine albumin levels using a dipstick kit.

Determination of Inflammatory Markers

The blood samples were collected through the cardiac puncture to estimate the serum levels of CRP, TNF-alpha, and IL-6.

Histopathological Examination

All the rats were euthanized at the end of eight weeks through a high dose of sodium pentobarbitone. Their kidneys were excised carefully without any damage and stored in 10% neutral buffered formalin after washing with phosphate buffer saline (PBS). The kidneys were then processed and embedded in paraffin. The kidneys were sectioned and stained with hematoxylin, eosin, and periodic acid Schiff base for histopathological observations. The sections were then analyzed for the degree of tubular and glomerular damage. The glomerular damage index (GDI) was calculated on a scale of 0 to 4 based on the degree of glomerulosclerosis, mesangiolysis, and mesangial expansion. Histopathological examination of the kidney was done according to Ozdemir O et al., as shown in table 1.[21]

Table 1: Scoring System for Histopathological Examination of Kidneys

0 – No light microscopy changes
1 - Minimal changes, >5 and <10 tubules in 5 Low power field (LPF) with vacuolar degeneration and cystic dilation of tubules
2 – Mild changes, > 10 and <15 tubules in 5 Low power field (LPF) with vacuolar degeneration and cystic dilation of tubules
3 – Moderate changes, >15 and <20 tubules in 5 LPF with vacuolar degeneration and cystic dilation of tubules
4 – Severe changes, >20 tubules in 5 LPF with vacuolar degeneration and cystic dilation of tubules or mesangial expansion

Statistical Analysis

The data is presented as Mean ± SEM for all the groups. It was analyzed by one-way ANOVA followed by Post hoc Dunnett's test, and post hoc Bonferroni's test was conducted to determine the statistical comparisons. The analysis was done using graph pad prism software, and $p \leq 0.05$ was considered statistically significant.

RESULTS

Ranolazine attenuates diabetes-induced body weight loss and hyperglycemia in rats

The change in the rats' body weights in all four groups is presented in Table 2 and Figure 2. Control rats showed a slight increase in body weight throughout the study period, but the weights of the diabetic control group rats significantly ($p < 0.001$) decreased owing to the induction of diabetes. At the end of eight weeks, body weight increased significantly in the ranolazine-treated group ($p \leq 0.01$) and metformin-treated group ($p \leq 0.001$) as compared to the diabetic control group. In diabetic rats, the blood glucose level was significantly high (~ 400 mg/dl) throughout the study period ($p < 0.001$). The diabetic rats in the ranolazine-treated group showed a significant reduction ($p \leq 0.001$) in blood glucose levels at the end of eight weeks compared to the diabetic control group. In contrast, the reduction in random blood glucose levels in a metformin-treated group ($p \leq 0.0001$) was significantly higher than in the ranolazine-treated group.

HbA1c % increased significantly in diabetic rats (p≤ 0.0001) compared to the normal control group. At the end of the eighth week, diabetic rats in the ranolazine-treated group (p≤ 0.0001) and metformin-treated group (p≤ 0.0001) showed a significant reduction in HbA1c % in contrast to the diabetic control group. Between the standard drug and test drug group, diabetic rats in the metformin-treated group showed a significant reduction in HbA1c % compared to the ranolazine-treated group.

Table 2. Effect of ranolazine on metabolic parameters and serum biochemistry on STZ induced diabetic rats assessed at the end of 8 week

Parameters	Groups			
	Normal Control	Diabetic Control	Metformin	Ranolazine
Body weight(g)	272.6±2.5	160.6 ± 2.0	242.6±1.47***	190.5±2.37**
Blood glucose(mg/dl)	109.1± 2.9	447.0± 17.29	114.3± 1.47 ****	159.3± 5.7***
HbA1c (%)	1.66 ± 0.39	9.41± 0.38	4.05 ± 0.3****	6.32 ± 0.27 **** #
Sr Creatinine(mg/dl)	0.74±0.34	3.50±0.34	0.78±0.4****	1.21±0.37****
Urine albumin	-	+++	+	+

The table represents metabolic parameters and serum biochemistry of the animals from four groups: Normal control group, Diabetic control(STZ 45mg/kg),Metformin treated group(STZ 45mg/kg + Metformin 180mg/kg/day),Ranolazine treated group(STZ 45mg/kg+90mg/dl/day). Values are expressed as mean +/- SEM(n=8) of the samples. Significant difference between control and diabetic groups:#p≤0.05,##p≤0.01,###p≤0.001.significant difference between diabetes group and metformin treated group, diabetes group and ranolazine treated group * p≤0.05,** p≤0.01,*** p≤0.001, **** p<0.0001

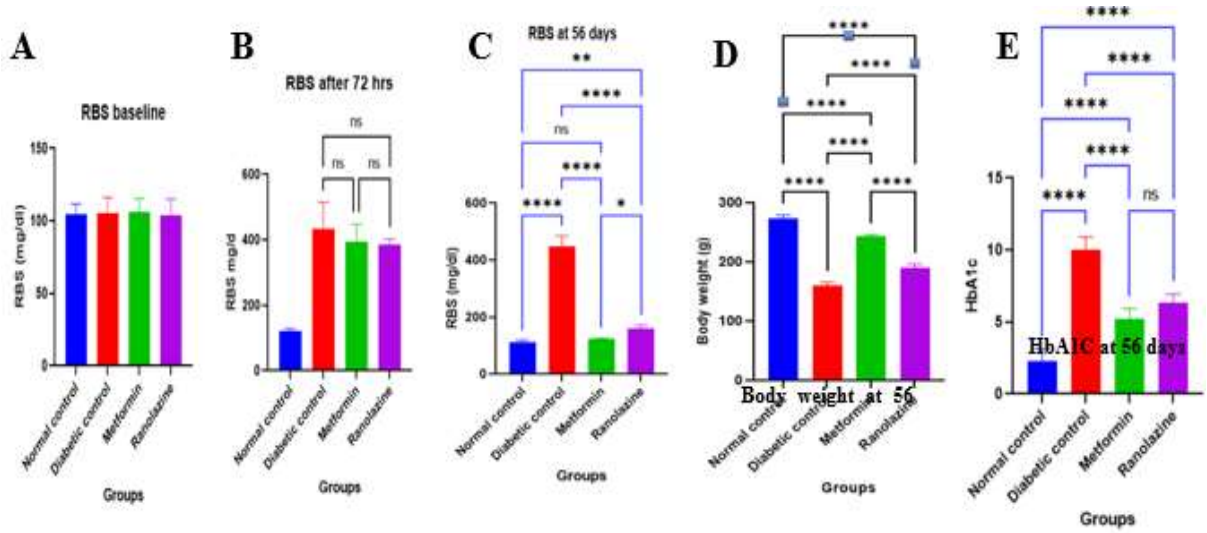

Figure 2: Effects of Ranolazine on metabolic parameters in rats with diabetic nephropathy. A, B, C Random Glucose (D) Bodyweight and (E) HbA1C were assessed. Data are presented as the mean ± standard error of the mean. *p < 0.05,***p< 0.001, ****p< 0.0001 vs normal control group and #p < 0.05, ## p<0.01, ###p< 0.001, ####p< 0.0001 vs Diabetic control group

Ranolazine suppresses inflammatory markers in diabetic rats

Given inflammation caused by diabetes and the associated nephropathy, we explored the effect of ranolazine on inflammatory markers in the serum. CRP, TNF-alpha, and IL-6 levels increased significantly in the diabetic group (P < 0.001) compared to the control group. At the end of the eighth week, diabetic rats in the ranolazine-treated group (p≤ 0.001) and metformin-treated group (p≤ 0.0001) showed a significant reduction in CRP, TNF -alpha, IL-6 levels as compared to the diabetic control group. Diabetic rats in the metformin-treated group showed a significant decrease in CRP, TNF -alpha, and IL-6 levels compared to the ranolazine-treated group, as shown in table 3.

Table 3

Effect of ranolazine on Inflammatory markers at the end of 8 weeks

Parameters	Groups			
	Normal Control	Diabetic Control	Metformin	Ranolazine
CRP	38.85±3.19	230.8±8.25	129.6±7.93****	135.9±2.37****
TNF -alpha	37.3±1.8	142.8±12.25	56.2±6.8****	78.45±2.37***
IL-6	89.31 ± 7.7	578.9 ± 24.6	247.28 ± 26.1****	295.03 ± 41.9***

Values are expressed as mean +/- SEM(n=8) of the samples. Significant difference between control and diabetic groups:#p≤0.05,##p≤0.01,###p≤0.001.significant difference between diabetes group and metformin treated group, diabetes group and ranolazine treated group * p≤0.05,** p≤0.01,*** p≤0.001, **** p<0.0001

Ranolazine suppresses serum creatinine and urine albumin in diabetic rats

Serum creatinine in the diabetic control group (p< 0.0001) showed a significantly higher value than in the normal control group. Diabetic rats in the ranolazine-treated group (p≤ 0.0001) and metformin-treated group (p≤ 0.0001) showed a significant decline in serum creatinine compared to the diabetic control group.

Spot urine samples were collected using a dipstick kit to estimate urinary albumin concentration. Albumin excretion of 30-150 mg/24 hours is considered microalbuminuria. The excretion level of urine protein >300 mg/dl is proteinuria in DNP. Normal control rats did not exhibit proteinuria (+++), whereas 50% of rats in the diabetic control group showed >300 mg/dl of urine albumin, and the remaining rats showed >100 mg/dl of urine albumin. Diabetic rats in the ranolazine-treated & metformin-treated groups showed <100 mg/dl albumin in their urine.

Ranolazine attenuates diabetic nephropathy

The Reno protective potential of ranolazine in diabetic rats is supported by a histopathological study. Renal cross-sections were examined using hematoxylin and eosin staining (Images: H&E stain under PAS 40x magnification). The extent of renal injury was assessed by morphometric analysis of glomerular disease, tubular damage, and interstitial fibrosis. Based on the current knowledge regarding histopathological changes in STZ-induced diabetic nephropathy in rats, we devised a scoring system based indirectly on a study by Ozdemir O et. al. [21]

Histopathological examination of the renal sections showed intense vacuolar degeneration and cystic dilatation of the tubules with a median score of 2 (in a range of 1-2) in the diabetic control group. In contrast, diabetic rats in the ranolazine- and the metformin-treated group were scored with a median score of 1 (in a range of 0-1), as shown in Table 1, suggesting that their kidney histology was close to normal with few changes. Diabetic-treated groups with ranolazine and metformin maintained near-normal renal architecture despite interstitial nephritis. Figure 2 illustrates the changes in the kidney histology of rats in all four groups.

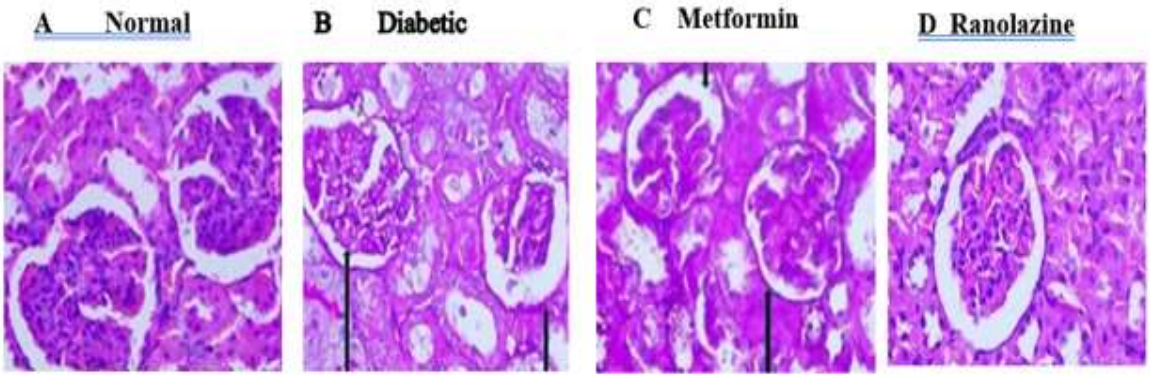

Fig 2: Histopathology images of kidney sections from A) Normal control rats showing normal interstitium with normal-appearing glomeruli and basement membrane, B) Diabetic control rats showing distorted glomeruli with thick basement membrane and nephritis, C) metformin-treated rats showing slightly distorted glomeruli and thick basement membrane, D) Ranolazine-treated rats showing mild interstitial nephritis with the slightly thick basement membrane.

DISCUSSION

Diabetic nephropathy (DNP) is mainly caused by the formation of advanced glycation end products and inflammation due to chronic hyperglycemia. As a result, any therapeutic intervention aimed at this pathogenesis would be the most effective treatment for DNP. Our study aimed to investigate the Renoprotective potential of ranolazine by ameliorating diabetic nephropathy in a rat model of streptozotocin-induced diabetes.

Diabetic nephropathy was induced in male Wistar rats through intraperitoneal administration of a single dose of STZ (45 mg/kg). Ranolazine-treated rats (n=8) were administered with ranolazine (90 mg/kg) orally every day for eight weeks. The results demonstrated that ranolazine prevents the progression of renal lesions (Fig. 2) in diabetic rats.

Histopathological examination of the kidney was analyzed by a scoring system derived from a study by Ozdemir O et al. Renal histopathology of rats in the diabetic control group reflected the median score of 2 (of range 1-2), indicating vacuolar degeneration, cystic dilatation of the tubule, moderate interstitial nephritis, distorted glomeruli, and basement membrane thickness. Histopathology of kidneys in ranolazine-treated rats showed mild interstitial nephritis with a slightly thickened basement membrane. It reflected the median score of 1 (of range 0-1), suggesting that kidney histology was close to normal with few changes and delayed the progression of renal damage. At the same time, ranolazine can decrease the RBG levels, reduce HbA1c %, and increase body weight along with a decrease in inflammatory markers CPR, IL-6, TNF-alpha, and a drop in serum creatinine and urine albumin levels. As Chronic hyperglycemia plays a major role in inducing severe renal lesions affecting renal functions and treatment with ranolazine indicated the importance of glycaemic control in controlling inflammation and oxidative stress in the management of diabetic nephropathy. This discovery is crucial as ranolazine might be a potent preventive drug for diabetic nephropathy and should be investigated thoroughly in future trials.

Streptozotocin (STZ) is widely used in studies investigating Diabetes Mellitus (DM), as it targets β-cells and reduces blood insulin levels, leading to hyperglycemia and mimicking DM pathology.[22] For the current study, diabetic nephropathy was induced in a rat model via STZ (45 mg/kg) for eight weeks to cause chronic hyperglycemia. Elevated glucose levels for eight weeks successfully induced renal lesions in rats that were similar in human patients with DNP.[23] In the present STZ-induced DNP model, the kidney damage was assessed via kidney function parameters; the presence of albumin in the urine is a primary indicator of kidney damage in the early onset of DNP. Kidney damage in diabetic rats was indicated by a considerable rise in proteinuria, serum creatinine, extracellular matrix deposition, and thickening of the glomerular basement membrane found eight weeks after the initiation of STZ-induced hyperglycemia. Treatment with ranolazine monotherapy ameliorated the majority of these renal dysfunctions and morphological changes, demonstrating that ranolazine treatment has preventive benefits in diabetic rats.

The major pathophysiology of diabetic nephropathy is due to persistently elevated blood glucose levels and the generation of advanced glycation products resulting in increased production of inflammatory cytokines and free radicals, leading to renal damage. Diabetic nephropathy advances with mesangial expansion caused by matrix deposition, thickening of the basement membrane, and the creation of Kimmelstiel–Wilson nodules in the glomeruli. As a result of the above-stated facts, it is reasonable to conclude that inflammation and oxidative stress are the primary mechanisms behind diabetic nephropathy. [24,25]

As hyperglycemia occupies a dominant position at the beginning of the ROS-mediated pathway, strict glucose modulation remains the vital therapeutic strategy. It is because it aids the amelioration of oxidative stress. [26,27,28] In the present study, the ranolazine-treated group showed a significant reduction in RBG compared to the diabetic control group. This result indicated that glycemic control is crucial for the ranolazine-mediated renoprotective effect. Furthermore, several preclinical and clinical research support the evidence of the anti-inflammatory efficacy of ranolazine, suggesting that ranolazine may have a novel mechanism of action in this setting.[29] Our results indicate that STZ-induced diabetic nephropathy in the diabetic control group is associated with increased levels of pro-inflammatory mediators (TNF-α, IL-6, and CRP). The current findings are consistent with the previous evidence. In chronic models of diabetic nephropathy, chemokines released from immune cells in the kidney were correlated with renal changes. Hyperglycemia activates inflammatory pathways, and prolonged inflammation exacerbates renal damage through sustained overexpression of pro-inflammatory factors that induce more inflammation and enhances other pathogenic mechanisms such as oxidative stress. High levels of CRP, TNF-α, and IL-6 partly mediated alterations in histopathological changes detected in diabetic nephropathy.[30] The anti-inflammatory effects of ranolazine are well established in various animal studies. It concluded that ranolazine improves TNF-α and 1L-1 levels.[12] We found that ranolazine significantly reduced CRP, TNF-α, IL- 6 levels compared to diabetic-controlled rats. And the findings of the current paper are in line with the existing evidence.

The results suggest that treatment with ranolazine exerts a renoprotective effect by controlling hyperglycemia-mediated inflammation and oxidative stress. The current study detected uncontrolled nephropathy in the diabetic control group, which was characterized by high glucose levels, proteinuria, and significantly elevated levels of CRP, IL-6, and TNF-α in the serum. The paper's results demonstrate that ranolazine reduces blood glucose levels and that reduced inflammation prevents the chronic pathogenesis of DNP. Therefore, the antidiabetic and anti-inflammatory effects caused by ranolazine are another important mechanism that protected the rats from progressing renal damage caused by chronic diabetes.

CONCLUSION

The study evidences the Renoprotective effects of ranolazine by ameliorating diabetic nephropathy in STZ-induced diabetic nephropathy in male Wistar rats. The successful induction of diabetic nephropathy in the rats was demonstrated by hyperglycemia, severe renal dysfunction, and increased expression levels of inflammatory cytokines. Ranolazine treatment significantly reduced blood glucose levels, protected renal functions, and retained near-normal renal morphology. The renoprotective role of ranolazine may be because of its glycaemic control, anti-inflammatory and anti-oxidative effect. However, further investigation is required to elucidate the underlying mechanism.

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