

Effect of combining an angiotensin-converting enzyme inhibitor and a vitamin D receptor activator on renal oxidative and nitrosative stress in diabetic rats

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ABSTRACT

Background: Diabetic nephropathy (DN) is a progressive and irreversible renal disease. Experimental researches have established the role of oxidative stress as a central factor in pathogenesis, onset, and advancement of DN. **Aims and Objectives:** To investigate the effect of the combined treatment with angiotensin-converting enzyme inhibitor, enalapril, and the specific vitamin D receptor activator paricalcitol, alone or in combination, using a diabetic rat model. **Materials and Methods:** Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg bw). The diabetic (D) rats were treated for 3 months as follows: diabetic control (DC) treated with vehicle (100 ml propylene glycol ip), enalapril treated group (EG; 25 mg/l in drinking water), paricalcitol treated group (PG; 0.8 mg/kg ip, 3 × week), or combined treatment group (CG) treated with both enalapril and paricalcitol with the same doses described above. A group of normal rats was served as control (N). Biochemical analysis was performed using an automatic biochemistry analyzer. Evaluation of oxidant/antioxidant balance and immunohistochemical localization of 3-nitrotyrosine (3-NT) in the kidney tissue was performed. **Results:** Combined treatment with both drugs was associated with significantly lower blood glucose, malondialdehyde, nitric oxide, levels and significantly higher levels of the antioxidant parameters more than those observed for monotherapy. Co-treatment led to additional improvement with negligible interstitial damage with no glomerular or tubular injury detected and strongly decreased 3-NT expression induced by diabetes. **Conclusion:** Co-treatment with both drugs exerts a synergistic protective effect against diabetic nephropathy by decreasing oxidative and nitrosative stress.

KEY WORDS: Enalapril; Paricalcitol; Oxidative and Nitrosative Stress; Diabetic Nephropathy; Rats

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INTRODUCTION

Diabetes is one of the most common noncommunicable diseases. It is the fourth or fifth leading cause of death in most high-income countries. Diabetes mellitus (DM) is a chronic debilitating condition that is rapidly increasing in prevalence worldwide, as a result of increases in obesity and changing patterns of diet and physical activity. Three of the world's top

10 countries with the highest prevalence (%) of diabetes are in the Middle East and North Africa region: Saudi Arabia, Kuwait, and Qatar.^[1] DM is complicated by cardiomyopathy, vasculopathy, neuropathy, nephropathy and retinopathy, and is responsible for all are major causes of morbidity and mortality.^[2] Diabetic nephropathy (DN) is the common cause leading to end-stage renal disease.^[3] Diabetic nephropathy is a progressive and irreversible renal disease characterized by the aggregation of extracellular matrix in glomerular mesangium and kidney interstitial tissue that ultimately leads to renal failure^[4] with an increased mortality rate.^[5] Experimental researches established the role of oxidative stress as a central factor in the pathogenesis, onset, and advancement of DN.^[6,7] Oxidative stress directly leads to injury and other pathways also cause injury via oxidative stress, and thus, inhibition of oxidative stress may constitute a focal point for multiple therapeutic synergies.^[8,9] Furthermore, reactive oxygen species (ROS) and other free radicals can directly induce injury. Oxidative stress activates pathogenic pathways such as renin-angiotensin-aldosterone system (RAAS), protein kinase C- β (PKC-B), and advanced glycation end-product (AGEs).^[10] In the kidney, continual over activation of the RAAS results in glomerular hypertension, fibrosis, and proteinuria, which leads to progressive renal damage.^[11] Angiotensin II activates NADPH oxidase, which leads to the superoxide ions formation.^[12] AGEs can induce ROS production and activate PKC by induction of oxidative stress in mesangial cell.^[13] Antioxidant effects of angiotensin-converting enzyme inhibitor (ACEI) may contribute to the ability of these agents to prevent DN. Indeed, enalapril (ACEI) has been reported to increase antioxidant enzyme activity in kidneys of rats with DM.^[14] One of the oxidation products that are proposed to lead to progression of DN is the 3-nitrotyrosine (3-NT).^[15] It is a measure of nitrosative stress that can be evaluated by assessing nitrated proteins by immunohistochemistry using antibodies against 3-NT.^[16] Enalapril increases superoxide dismutase (SOD) activity and prevents accelerated O₂ and 3-NT production in renal cortex during *in vitro* acute exposure to high glucose levels.^[17] These observations suggest that in addition to its antihypertensive and antiproteinuric effects, enalapril and other ACEIs may suppress renal cortical 3-NT levels through a mechanism involving a reduction in Ang II-stimulated NADPH oxidase activation, leading to reduced O₂ production and enhanced O₂ degradation resulting from SOD activation. Some beneficial effects of ACE inhibition may reflect prevention of DM-induced posttranslational modification events involving nitration of tyrosine residues. The vitamin D receptor (VDR) is a ligand-activated transcription factor that can modify the expression of target genes after activation by a specific ligand.^[18] The glomerulosclerosis index and urine albumin excretion in rats with subtotal nephrectomy decreased by the endogenous VDR ligand, calcitriol.^[19] Mice were protected from developing DN by the combination of a VDR activator and an ACE inhibitor.^[20] Paricalcitol (a selective VDR activator) showed a significant reduction of proteinuria, independently of glomerular filtration rate, blood pressure, or ACE inhibition in chronic kidney disease

(CKD) patients after 23 weeks of therapy.^[21] It was found that the combination therapy of paricalcitol and the enalapril had an added benefit in ameliorating the progression of renal insufficiency by suppressing inflammatory pathways in the uremic rat model.^[22] Regarding the oxidation markers, the reduction in malondialdehyde (MDA) levels by paricalcitol was also reported in an animal model, and it was postulated that the reason could be downregulation of RAAS by vitamin D analogues.^[23] The antioxidant effect of paricalcitol is observed with the reduction of carbonyl groups and nitrites. Nitrites reduction may be due to downregulation of endothelial nitric oxide synthetase and inducible nitric oxide synthetase (iNOS) activities, which would respond to an antioxidant and anti-inflammatory effect of VDR activation.^[24] The reduced production of nitrites, MDA, and carbonyl groups could also be a result of the increased antioxidant elements such as reduced glutathione (GSH), thioredoxin, and SOD, scavenging excess ROS before excessive oxidation takes place.

To the best of our knowledge, the mechanisms behind the actions of co-treatment with paricalcitol and enalapril on the renal oxidative and nitrosative stress in the diabetic rats are not fully understood. Therefore, the aim of this study was to investigate the ability of the combination of ACE inhibitor, enalapril, and the specific vitamin D receptor activator (VDRA), paricalcitol to prevent diabetic nephropathy by affecting the renal oxidative and nitrosative stress using the streptozotocin-induced diabetic rat model.

MATERIALS AND METHODS

Animals

Male Wistar rats ($n = 50$, 140–180 g) were housed in cages with 12/12 h light/dark cycle at 21 ± 2 °C.

Induction of diabetes. The animals were acclimatized for 7 days before the study and were free to access water and standard rat chow throughout the experiment. The animals were fasted for 24 h before the induction of diabetes. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St Louis, MO) (50 mg/kg body weight), freshly dissolved in citrate buffer (0.1 M, pH 4.5), whereas control rats were injected with vehicle buffer only. Blood samples were obtained from the tail vein of the animals at 72 h after STZ injection, and fasting blood glucose levels was determined with a glucose strip test in a glucometer (Easy Gluco Blood Glucose Monitoring System; Infopia, Korea). Rats with fasting blood glucose levels above 250 mg/dl were used as diabetic animals.

Experimental design. A total of 50 rats were used and were divided into five groups of 10 rats each. In this study, diabetic (D) rats were treated as follows: D + vehicle (DC) treated with vehicle (100 ml propylene glycol ip), D + enalapril (Hiral Labs Ltd, India) (EG; 25 mg/l in drinking water), D + paricalcitol (MedChemexpress, Princeton, NJ) (PG; 0.8 mg/kg ip, 3 weeks), or D + enalapril + paricalcitol (CG). A group of normal rats also served as control. The rats were treated for 3 months. All animals were fed a high-phosphorus rodent diet containing

0.9% phosphorus and 0.6% calcium. Approximately 20 h after the last treatment, the rats were killed by exsanguinations via the dorsal aorta and blood was taken for analysis. Portions of kidney were taken for analysis. Others were snap-frozen in liquid nitrogen and stored at -80 °C until later analysis.

Biochemical Analysis

Fasting blood samples were obtained from the tail veins. Blood glucose was measured by a glucometer (Roche, Basel, Switzerland). Serum levels of urea and creatinine were detected using an automatic biochemistry analyzer (Hitachi, Inc., Japan).

Evaluation of Oxidative Stress

Preparation of renal tissue: Cortex and medulla were washed thoroughly with ice-cold saline, 10%(w/v); each tissue was homogenized separately in a Potter-Elvehjem homogenizer in ice-cold 50mM phosphate buffer (pH 7.4) containing mammalian protease inhibitor cocktail. The homogenates were centrifuged at $10,000 \times g$ for 30min at 4 °C. The supernatants were used for measuring the enzyme activities. A portion of the supernatant was used for the determination of total protein concentration by Bradford method^[25] using bovine serum albumin as standard.

Renal Total Antioxidative Capacity (TAC)

The renal Total antioxidant capacity of plasma was measured using a total antioxidant capacity kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Plasma was allowed to reduce Cu^{2+} for 1.5 h at room temperature. Reduced Cu^{+} was chelated with a colorimetric probe and absorbance was measured at 570 nm. Results were expressed as Trolox equivalent according to a Trolox standard curve.

Catalase Assay

Renal catalase (CAT) activity was assayed with a catalase assay kit (ab83464; Abcam) at 25 °C by Aebi method, which is based on the disappearance of H_2O_2 from a solution containing 30mmol/l H_2O_2 in 10mmol/l potassium phosphate buffer (pH 7) at 240nm.^[26] Results were expressed as μmol of H_2O_2 consumed/min/mg protein.

Glutathione Peroxidase Assay

Renal glutathione peroxidase (GPx) activity was assessed using a Glutathione Peroxidase Assay Kit (ab102530; Abcam) by a method previously described.^[27] The results were expressed as units/mg protein.

Superoxide Dismutase Assay

Superoxide dismutase activity in kidney homogenates was measured using a Superoxide Dismutase Activity Colorimetric Assay Kit (Abcam), by a competitive inhibition assay using xanthine-xanthine oxidase system to reduce nitroblue tetrazolium, a previously reported method.^[28] Results were expressed as U/mg protein.

MDA Level

The MDA contents in the kidney and NRK-52E cells were measured by thiobarbituric acid method^[29,30] with commercially available kits (MDA Assay Kit; Abcam) following the manufacturer's instruction. The samples were analyzed using 450-nm dual wavelength to eliminate the influence of glycation and part of other lipidic aldehydes. All measurements were performed in triplicate, and the results were expressed as nmol /100 mg tissue/h.

NO Level

By the determination of the stable NO end products, nitrite and nitrate, NO level was estimated spectrophotometrically. The level of serum nitrite was determined using the Griess reagent according to the method described by Hortelano et al.^[31] The Griess reagent, a mixture (1:1) of 1% sulfanilamide in 5% phosphoric acid and 0.1% 1-naphtylethylenediamine, gives a red-violet diazo color in the presence of nitrite. The color intensity was measured at 540 nm. Data were expressed in $\mu\text{mol/l}$ using a sodium nitrite calibration graph.

Immunohistochemical Localization of 3-Nitrotyrosine

For immunohistochemical analysis, paraffin-embedded kidney tissues were sectioned (5 μm) and transferred to positively charged slides. Samples were treated with H_2O_2 (4.5%) to quench/inhibit endogenous peroxidase. After blocking, the sections were reacted with anti-3-NT antibody (anti-3-nitrotyrosine antibody [11C2] (ab53232); Abcam) for 1h at room temperature. After extensive washing with phosphate-buffered saline, the sections were incubated with antibody peroxidase conjugated for 1h and finally incubated with diaminobenzidine for 30min. Quantitative image analyses were performed with an image analysis software (Image-Pro Plus, version 6.0; Media Cybernetics Inc., Bethesda, Maryland, MD, USA). The software determines densitometry mean values of selected tissue regions. Thus, 10 fields/rats were randomly selected, and the intensity of the 3-NT immunostaining was determined.

Statistical Analysis

Data were expressed as the mean \pm SD. Each variable was assessed for a normal distribution using the Kolmogorov-Smirnov test. Statistical differences between the groups were identified using one-way analysis of variance. All statistical results were based on two-sided tests. Data were analyzed using Package for Social Sciences (SPSS) software for Windows (version 22.0; SPSS Inc., Chicago, IL, USA). A P value <0.05 was regarded as statistically significant.

RESULTS

Effect of Treatment on Renal Function and Glycemic Status

This study shows that diabetic rats with no treatment had a significantly higher levels ($P < 0.05$) of urea, creatinine, fasting blood glucose (FBG), postprandial BG, and homeostasis model assessment of insulin resistance (HOMA-IR) compared to normal rats. Moreover, diabetic rats had a significantly lower insulin and c-peptide than normal group. Treatment with enalapril or

Table 1: Effect of enalapril, paricalcitol, and combined treatment with both drugs on renal function and glycemic status

Parameters	Groups				
	Normal	DC	EG	PG	CG
Urea (mg/dl)	32.82 ± 12.74	90.83 ± 21.09 ^a	43.04 ± 4.54 ^{a,b}	58.17 ± 16.01 ^{a,b,c}	47.30 ± 7.08 ^b
Creatinine (mg/dl)	.65 ± .10	1.62 ± .25 ^a	.78 ± .16 ^b	.82 ± .17 ^b	.76 ± .55 ^b
FBG (mg/dl)	65.60 ± 18.10	220.07 ± 69.83 ^a	135.25 ± 21.85 ^{a,b,c}	144.67 ± 19.86 ^{a,b,c}	106.00 ± 4.58 ^b
Postprandial BG (mg/dl)	95.17 ± 16.35	240.00 ± 41.01 ^a	192.75 ± 61.60 ^{a,b,c}	168.33 ± 71.41 ^c	80.50 ± 10.63 ^b
Insulin(ng/ml)	2.63 ± 0.31	0.61 ± 0.52 ^a	1.53 ± 0.42 ^{b,c}	1.41 ± 0.54 ^{b,c}	1.64 ± 0.56 ^b
C-peptide (ng/ml)	4.73 ± 0.72	0.78 ± 0.35 ^a	3.23 ± 1.17 ^{b,c}	4.78 ± 0.27 ^b	4.20 ± 0.40 ^b
HOMA-IR	1.58 ± 0.29	4.56 ± 1.16 ^a	1.87 ± 0.29 ^{b,c}	1.77 ± 0.50 ^b	1.41 ± 0.19 ^b

Data are expressed as mean ± SD, N = 10 animals.

DC, diabetic control; EG, enalapril treatment group; PG, paricalcitol treatment group; CG, combined treatment group; FBG, fasting blood glucose; postprandial BG, 2-h blood glucose after oral glucose loading (3 g/kg bw); HOMA-IR, homeostasis model assessment of insulin resistance; GSH, reduced glutathione; GST, glutathione S-transferase; MDA, malondialdehyde.

^aSignificantly different from control group, ^bsignificantly different from DC group, ^csignificantly different from combined treatment.

Table 2: Effect of enalapril, paricalcitol, and combined treatment with both drugs on oxidative stress parameters

Parameters	Groups				
	Normal	DC	EG	PG	CG
MDA (nmol /100 mg tissue/h)	17.38 ± 1.66	32.92 ± 5.50 ^a	22.13 ± 8.32 ^{b,c}	20.83 ± 4.17 ^{b,c}	18.59 ± 6.47 ^b
NO (µmol/l)	5.00 ± 0.25	20.88 ± 1.66 ^a	7.16 ± 0.34 ^{b,c}	7.96 ± 0.32 ^{b,c}	5.70 ± 0.54 ^b

Data are expressed as mean ± SD, N = 10 animals.

DC, diabetic control; EG, enalapril treatment group; PG, paricalcitol treatment group; CG, combined treatment group; MDA, malondialdehyde; NO, nitric oxide.

^aSignificantly different from control group, ^bsignificantly different from DC group, ^csignificantly different from combined treatment.

Table 3: Effect of enalapril, paricalcitol, and combined treatment with both drugs on antioxidant parameters

Parameters	Groups				
	Normal	DC	EG	PG	CG
GSH (nmol/100 mg tissue)	60.97 ± 23.86	28.99 ± 10.18 ^a	32.36 ± 6.69 ^{a,c}	31.24 ± 7.07 ^{a,c}	42.90 ± 14.76 ^{a,b}
GST activity (U/100 mg tissue)	125.40 ± 33.34	75.53 ± 15.53 ^a	116.80 ± 25.92	106.75 ± 5.41 ^c	119.60 ± 17.27
GPx (U/mg protein)	29.12 ± 2.82	19.82 ± 2.01 ^a	26.22 ± 2.07 ^b	25.06 ± 2.07 ^b	30.03 ± 3.07 ^b
CAT (µmol of H ₂ O ₂ consumed/min/mg protein)	6.47 ± 0.52	2.98 ± 0.26 ^a	5.23 ± 0.61 ^{b,c}	5.05 ± 0.55 ^{b,c}	6.77 ± 0.62 ^b
SOD (units/mg protein)	4.79 ± 0.46	2.24 ± 0.20 ^a	3.90 ± 0.43 ^{b,c}	3.04 ± 0.41 ^b	4.81 ± 0.56 ^b
TAC (µmol Trolox Eqv/mg protein)	2.30 ± 0.21	1.32 ± 0.43 ^a	1.93 ± 0.11 ^{b,c}	2.01 ± 0.14 ^{b,c}	2.27 ± 0.18 ^b

Data are expressed as mean ± SD, N = 10 animals.

DC, diabetic control; EG, enalapril treatment group; PG, paricalcitol treatment group; CG, combined treatment group; GSH, reduced glutathione; GST, glutathione S-transferase; GPx, glutathione peroxidase; CAT, catalase; SOD, super oxide dismutase; TAC, total antioxidant capacity.

^aSignificantly different from control group, ^bsignificantly different from DC group, ^csignificantly different from combined treatment.

paricalcitol ameliorated such effects. Combined treatment with both drugs was associated with lower urea compared to treatment paricalcitol alone but it did not differ significantly from enalapril treatment group. In addition, combined treatment was associated with significantly lower FBG and postprandial BG levels in this group than other treatment groups. In addition, combined treatment was associated with significantly lower FBG

and postprandial BG levels and HOMA-IR values than that seen in single treatment groups.

Effect of Treatment on Oxidative Stress Parameters

Table 2 shows that diabetic rats with no treatment have a significantly higher MDA and NO compared to normal rats

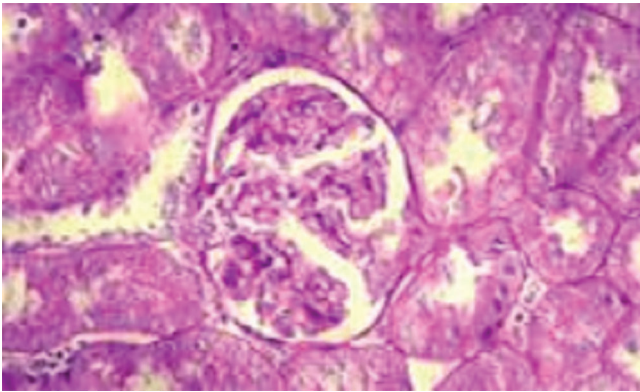


Figure 1: Section in kidney tissue belongs to normal control rat showing normal histology of the kidney.

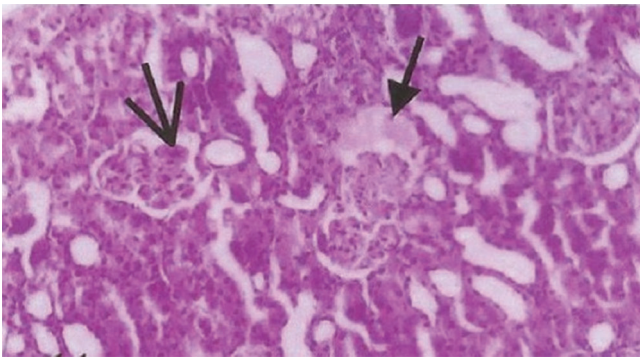


Figure 2: Light microscopic examination of kidneys from diabetic animals revealing diabetic glomerulopathy characterized by thickening of the glomerular basement membrane, mesangial matrix expansion, arteriolar hyalinosis, and large hyaline proteinaceous droplets.

($P < 0.05$). Treatment with enalapril or paricalcitol has ameliorated such effects. Also, combined treatment significantly decreased ($P < 0.05$) these oxidative stress parameters more than those seen in other treatment groups.

Effect of Treatment on Antioxidant Parameters

Table 3 shows the effect of treatment on antioxidant parameters. All antioxidant parameters are significantly ($P < 0.05$) decreased in diabetic group without treatment compared with normal rats. Treatment with enalapril or paricalcitol has ameliorated such decrease. Also, combined treatment significantly elevated ($P < 0.05$) the level of these antioxidant parameters more than those seen in other treatment groups.

Renal Histology

Figure 1 demonstrates that a section in kidney tissue belongs to normal control rat, showing normal histology of the kidney (hematoxylin and eosin (H&E), 400 \times) with no lesions detected. Figure 2 shows a light microscopic examination of kidneys from diabetic animals revealing diabetic glomerulopathy

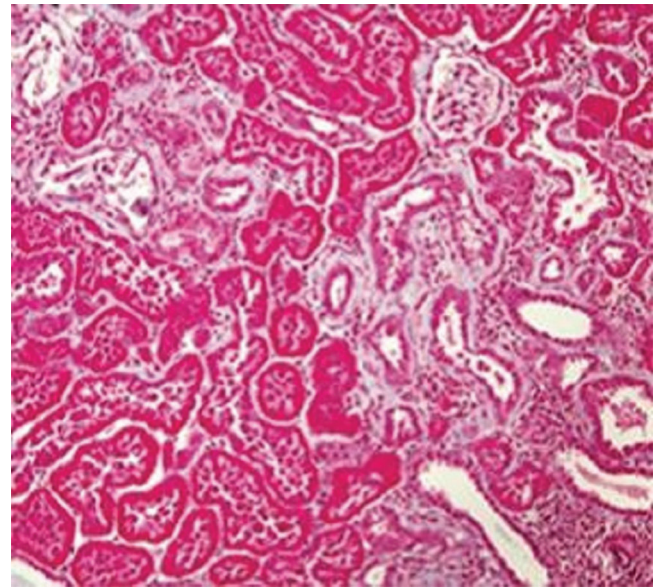


Figure 3: Representative microphotographs of Masson's trichrome-stained section from enalapril-treated diabetic rats after 3 months of treatment. Magnification 200 \times .

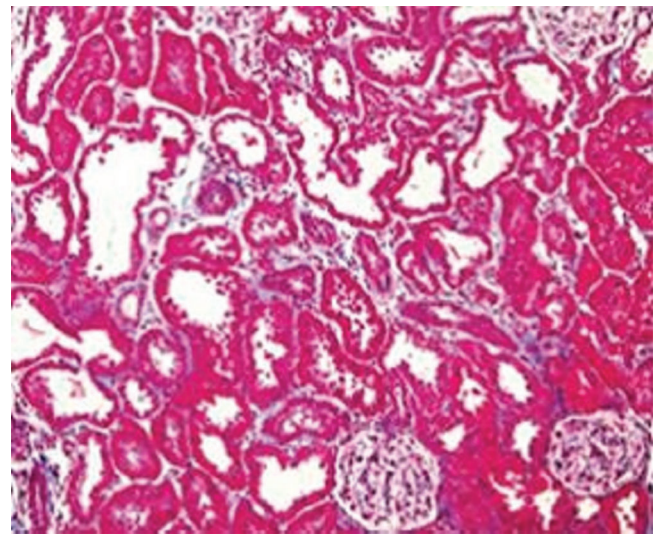


Figure 4: Representative microphotographs of Masson's trichrome-stained section from paricalcitol-treated diabetic rats after 3 months of treatment. Magnification 200 \times .

characterized by thickening of the glomerular basement membrane, mesangial matrix expansion, arteriolar hyalinosis, and large hyaline proteinaceous droplets (arrow) within the glomeruli (H&E, 200 \times), occluding capillary loops and attached outside the Bowman's capsule. Figure 3 is representative microphotographs of Masson's trichrome-stained section from enalapril-treated diabetic rats after 3 months of treatment. Sections show only moderate interstitial fibrosis, scant chronic interstitial

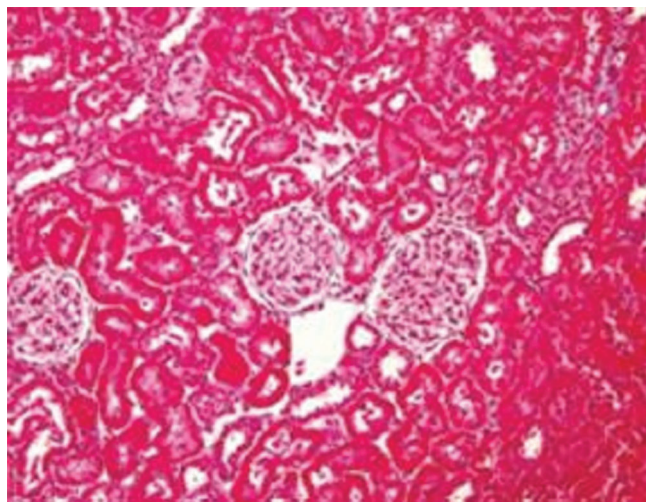


Figure 5: Representative microphotographs of Masson's trichrome-stained section from diabetic rats co-treated with enalapril and paricalcitol after 3 months of treatment. Magnification 200 \times .

inflammation, and tubular dilatation. Similar results were seen with paricalcitol treatment [Figure 4]. Co-treatment with both drugs caused additional improvement with negligible interstitial damage, and no glomerular or tubular injury were detected in this group [Figure 5].

Immunohistochemistry

In Figure 6, 3-NT expression is determined by immunohistochemistry in the inner part of the cortical kidney before and after induction of diabetes. Partial prevention by drug treatment with enalapril, paricalcitol, and combined treatment with both drugs can be detected. There is no 3-NT immunostaining in the kidney of normal rat. In contrast, there is a strong 3-NT expression in the necrotic cells from the straight portion of the proximal convoluted tubules (arrows) in diabetic group without treatment. In enalapril or paricalcitol only treatment, less 3-NT expression is present. The administration of combined treatment with enalapril and paricalcitol strongly decreases 3-NT expression induced by diabetes.

DISCUSSION

This study reports the effects of treatment with enalapril, an ACEI, and paricalcitol, a VDRA, alone or in combination, on the progression of renal impairment through their protective effects against renal oxidative and nitrosative stress in diabetic rats. The present study revealed the efficacy of this co-treatment in reversing deleterious effect of diabetic oxidative and nitrosative stress, which was documented by the amelioration recorded in kidney function tests and histopathology. We show that treatment with enalapril improved the kidney functions as proved by decreasing serum urea and creatinine

levels. Enalapril prevented glomerular sclerosis and reduced interstitial infiltration of mononuclear cells into the kidney. Paricalcitol also ameliorated and prevented glomerulosclerosis. This VDRA also reduced interstitial infiltration of mononuclear cells into the kidney. The combination of both drugs markedly prevented interstitial infiltration than either drug alone. A previous study revealed similar results with enalapril and paricalcitol treatment on glomerulosclerosis and interstitial infiltration of mononuclear cells.^[32] The effect of paricalcitol could be due, at least in part, to its effects on renin as vitamin D is known to be a negative regulator of *renin* gene expression.^[33] ACEIs have been shown to improve glomerular hemodynamics and structures in both human and experimental DN.^[34] It is well known that oxidative stress and inflammation are implicated in both renal and cardiovascular diseases. Oxidative stress can result from an excess of free radicals/ROS, a decrease in antioxidants, or a combination of both. Angiotensin II is known to induce oxidative stress by activating NADPH oxidase, which leads to the generation of superoxides.^[35] Oxidative stress may be both the cause and the result of tissue damage, and a primary and a secondary source of diabetic pathology.^[36] The recorded rise in tissue concentration of MDA, an index of endogenous lipid peroxidation, has been also reported by Turk et al.^[37] in patients with diabetes, and Kim et al.^[38] in diabetic rats reflecting increased state of oxidative stress. Both oxidative stress and AGEs result in nuclear factor- κ (NF- κ) activation. Concerning the renal antioxidant status, the current study revealed increased oxidative stress due to diabetes, which was evidenced by increased tissue concentration of MDA, NO, and depletion of antioxidant enzymes concentration. This was accompanied by significant glomerular pathology, namely glomerular sclerosis or glomerular atrophy, and accumulation of proteinous material in Bowman's space together with thickening of Bowman's capsule membrane. The reported oxidative stress resulted from hyperglycemia-induced increases in glucose autooxidation, protein glycation, and the subsequent oxidative degradation of glycated protein, leading to enhanced production of ROS.^[39] In type 2 diabetes mellitus (T2DM), cholecalciferol was found to decrease plasma glucose in obese Wistar rats.^[40] As shown in the present study, the combined treatment with both drugs ameliorated the increase in the levels of FBG, postprandial BG and HOMA-IR, and elevated insulin and c-peptide levels. Several potential mechanisms may account for the action of vitamin D on glucose and insulin metabolism.^[41] VDR stimulates insulin secretion, insulin receptor expression and insulin responsiveness,^[42] and oral cholecalciferol increased post-glucose load insulin secretion in patients with T2DM.^[43] Indirectly, VDR activation may reverse the reduced insulin sensitivity associated with increased parathyroid hormone activity.^[44] However, some studies did not show consistent results.^[43] We found that an additional evidence of the renoprotective effect of the combined therapy of enalapril and paricalcitol against oxidative stress induced by diabetes is the decrease in the level of MDA, which is a marker of oxidative stress and is one of the final products of polyunsaturated fatty acids peroxidation in the cells.^[45]

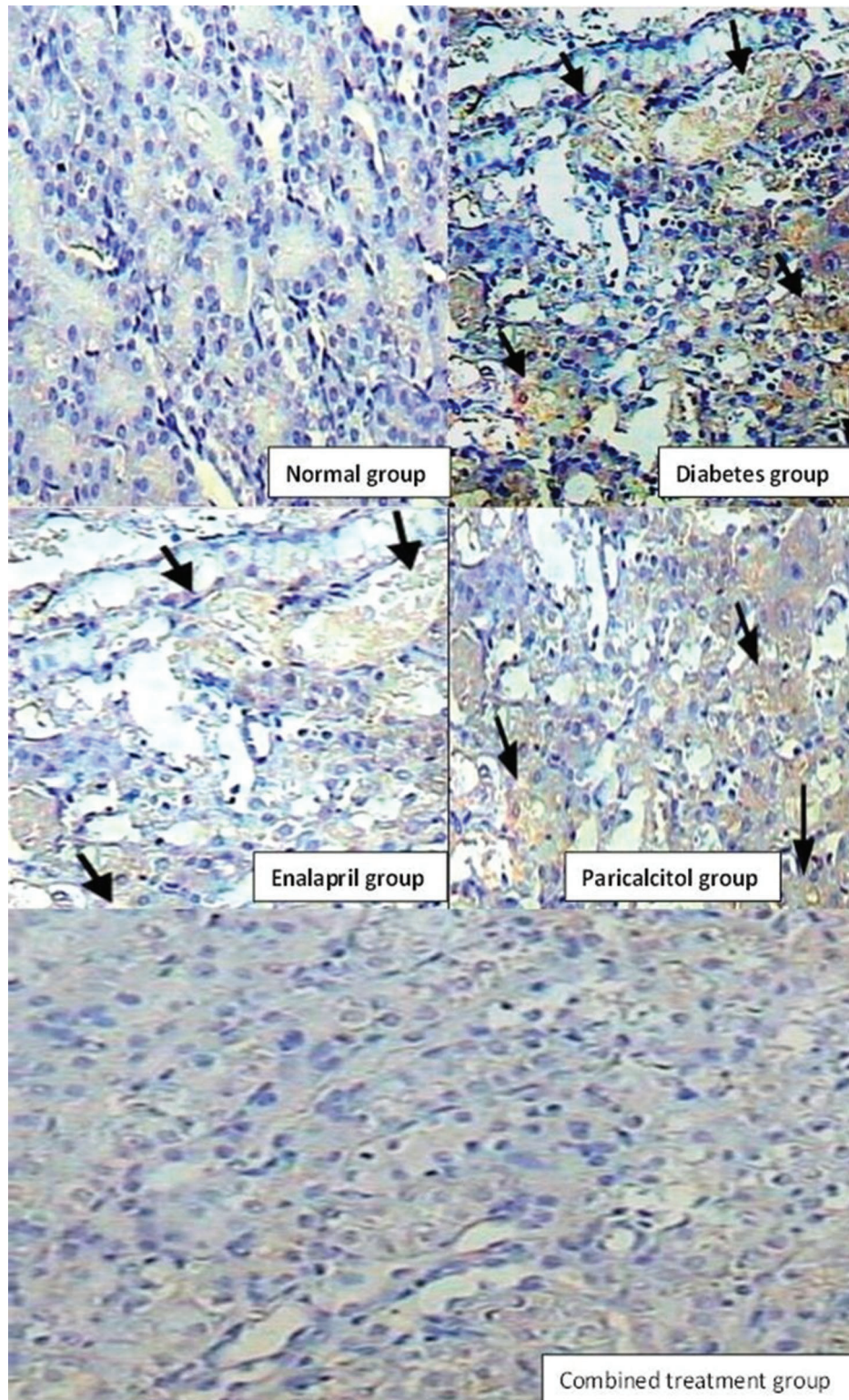


Figure 6: Nitrotyrosine (3-NT) expression determined by immunohistochemistry in the inner part of the cortical kidney before and after induction of diabetes and its partial prevention by drug treatment with enalapril, paricalcitol, and combined treatment.

Continual high levels of NO result in tissue damage, whereas low levels are necessary for various cell functions and to protect organs from ischemic damage. NO is known to occur at higher levels in an oxidative environment and is activated during inflammation by inflammatory cytokines.^[46] Our study data showed a significant inhibition of diabetes-induced increase in NO by monotherapy with enalapril or paricalcitol, and that co-treatment with both compounds resulted in detected inhibition of NO. This may be owing to inhibition of iNOS induction by these two drugs as renal iNOS expression can be increased in response to an increase in ROS.^[47] GSH is a major endogenous antioxidant produced by cells that neutralizes free radicals and ROS and maintains the active states of vitamins C and E. GSH has a fundamental role in protein synthesis and DNA synthesis and repair as well as in many other functions. Every system in the body is dependent on the state of the GSH system. Decreases in GSH levels can impair a cell's defense against ROS and result in oxidative injury.^[48] We measured the level of GSH and showed that it was decreased in diabetic rats without treatment, whereas treatment with either enalapril or paricalcitol inhibited the decrease in GSH. We also analyzed the activity of GPx, the other enzyme in the GSH redox cycle. GPx catalyzes the reduction of peroxides, including those formed during the production of GSH. We found that untreated diabetic rats showed a significant decrease in GPx activity but that enalapril or paricalcitol prevented this decrease. When given together, these two compounds had a more marked effect. Although nitric oxide is a simple inorganic radical showing diverse physiological functions, including the regulation of neurotransmission and vascular tone, yet it could react with superoxide yielding peroxynitrite, which is a potent nitrating and oxidizing agent that can nitrate and oxidize various biomolecules, such as thiols, lipids, carbohydrates, and nucleic acids.^[49] SODs scavenge superoxide by catalyzing the dismutation of superoxide to H₂O₂ and O₂. Mn-SOD is mitochondrial whereas CuZn-SOD is cytosolic.^[50] These two enzymes are considered to be the first line of defense against oxygen radicals in cells. Our reported results concerning the decrease in SOD during diabetes agreed with those of Godin et al.^[51] who showed a decrease in Cu-Zn SOD activity in renal tissues during diabetes; however they contradicted with those of Kakkar et al.^[39] and Limaye et al.^[52] who showed either no change or an increase in SOD activity in renal tissues of diabetic rats. Also a previous study revealed that superoxide anion production was accelerated twofold in renal cortical slices from diabetic rats, with an associated 50% increase in SOD activity.^[53] Also, enalapril or paricalcitol prevented this decrease and when given together; these two compounds had a more marked effect. Similarly, the present study showed a diabetes-induced decrease in glutathione *S*-transferase (GST) enzyme activities. GST belongs to a superfamily of multifunctional isoenzymes playing a vital role in the detoxifying mechanisms of drugs and xenobiotics by preventing the binding of reactive metabolites to cellular proteins and modulating the by-products of oxidative stress

by catalyzing the conjugation of electrophilic moieties to GSH.^[54] Treatment with enalapril, paricalcitol, or both seemed to restore activities of these group of enzymes to the activities recorded for the control group. Recent studies have suggested that increased oxidative and nitrosative stress is involved in the pathogenesis of diabetic microvascular injury in retinopathy, nephropathy, and neuropathy.^[55] Increased oxidative stress and nitrotyrosine formation have also been shown both in kidneys of diabetic animals^[54] and in biopsy specimen from patients with DN,^[55] suggesting pathogenetic role in the development of this complication. Recent studies in experimental animals have indicated that hyperglycemia stimulates the production of nitric oxide, which reacts with superoxide anion to form peroxynitrite, damaging the endothelium and perineurium. The increased ROS in the kidney, especially the superoxide radicals, react with NO to form peroxynitrite, which in turn binds to tyrosine and other protein residues, yielding highly cytotoxic compounds such as nitrotyrosine, which is a measure of ONOO⁻ (peroxynitrite) in the renal and other vascular tissues.^[56] Enhanced accumulation of nitrotyrosine in tissues in diabetes is thus at least in part an index of increased peroxynitrite generation, which in turn may reflect increased generation of NO, superoxide, or both.^[57] Nitrotyrosine accumulates in the kidney of rodents with STZ or genetic diabetes,^[58] consistent with enhanced renal production of NO and/or superoxide.^[57] Genetic overexpression of SOD1 in diabetic mice suppresses accumulation of nitrotyrosine in glomeruli to levels observed in nondiabetic, wild-type mice, and also attenuates renal injury in the diabetics. Thus, a reduction in the availability of superoxide to react with NO may be renoprotective in diabetes, in part by reducing the formation of the toxic-reactive intermediate peroxynitrite.^[58] Our study confirms an increase in renal nitrotyrosine expression in diabetes, such increase was ameliorated by monotherapy and the effect was marked by combined treatment as reported by decreased staining. As shown in the present study, the TAC level, which represents the antioxidant enzymes, macromolecules, and an array of small molecules,^[59] was significantly decreased in the diabetic group as compared to normal rats. Co-treatment was associated with marked increase in the TAC level, which reflects the modulation of oxidative stress associated with diabetes. The significant antidiabetic activity of the two drugs may be due to inhibition of the free radical generation and subsequent tissue damage induced by STZ or due to the enhancement of plasma insulin effect by either its increase in pancreatic secretion of insulin from existing beta cells or its release from bound form, as indicated by significant improvement in glucose level.

CONCLUSIONS

In summary, our data confirm that similar to enalapril, paricalcitol can suppress the progression of renal failure

by ameliorating glomerulosclerosis, interstitial infiltration, and oxidative stress. The effects of paricalcitol may be amplified when an ACEI is added because co-treatment with both compounds seems to have an additive effect on ameliorating diabetes-induced changes in MDA, NO, and SOD; peroxidase activity; catalase; total antioxidant capacity; and renal histomorphometry. As we have shown previously, the action of paricalcitol is probably, at least in part, via decreasing the expression of nitrotyrosine. Combination therapy with ACEI, or likely paricalcitol, may represent a novel and beneficial therapeutic strategy for suppressing the progression of CKD and ameliorating oxidative and nitrosative stress.

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