The effect of nitro-oleic versus losartan in diabetic nephropathy: modulation of parathyroid hormone related protein

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DOI: 10.5603/FM.a2018.0057

Article type: ORIGINAL ARTICLES

Submitted: 2018-05-03

Accepted: 2018-05-18

Published online: 2018-07-03

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The effect of nitro-oleic versus losartan in diabetic nephropathy: modulation of parathyroid hormone related protein

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Abstract:
Background: Parathyroid hormone related protein (PTHrP) involvement in the mechanisms related to angiotensin II (Ang II)-induced renal injury has become an emerging concern. The current study was thus designed to compare the possible preventive and therapeutic effect of Ang II antagonists; losartan and nitro-oleic (NO2-OA) acid on diabetic nephropathy (DN), and evaluate their effect on PTHrP modulation as well as on the functional and histopathological parameters in the kidney of diabetic rats.

Methods: Forty eight adult male Sprague Dawley rats were divided into control group, DN group, pre-diabetic nephropathy (pre-DN) losartan group, pre-diabetic nephropathy nitro-oleic acid (pre-DN NO2-OA) group, post-diabetic nephropathy (post-DN) losartan and post-diabetic nephropathy nitro-oleic acid (post DN-NO2-OA) groups. At the end of the study, systolic blood pressure (SBP), serum fasting glucose, glomerular filtration rate (GFR), urea, urea albumin excretion (UAE), serum angiotensin, renal PTHrP gene expression and correlations between PTHrP and SBP, serum glucose, Ang II and kidney functions were evaluated. Histological examination, Masson’s trichrome, Periodic acid shift staining as well as morphometric analysis and histopathological scoring for tubular and glomerular parameters have been carried out.

Results: Prophylactic losartan and NO2-OA were associated with improvement in SBP, serum glucose, urea, GFR, UAE, with reduction in serum Ang II and PTHrP overexpression observed in diabetic kidney. Treatment with losartan and NO2-OA
showed the same effect except that post-DN NO2-OA showed no significant effect regarding kidney function. Strong correlations were observed between PTHrP and SBP, serum glucose, Ang II and kidney functions. Histopathological results revealed obvious improvement in glomerulosclerosis, vascular and tubular injury parameters in prophylactic groups especially with losartan.

**Conclusions:** Both pre and post-DN losartan, NO2-OA may have a potential role in protection and regression of DN through reduction of PTHrP overexpression.

**Key words:** diabetic nephropathy, losartan, nitro-oleic acid, angiotensin II, PTHrP

**Introduction**

The treatment guidelines for DN always focus on slowing the progression of nephropathy by controlling glucose levels and decreasing blood pressure. However, this remains always suboptimal. Thus, it’s become more important to focus on novel molecular mechanisms and treatment modalities [1]. One of those newly evolving lines is the renal interaction between the renin-angiotensin-aldosterone system (RAS), parathyroid hormone related protein (PTHrP) and AngII in the kidney [2]. Current concepts indicate that PTHrP is a regulating factor, being more similar to other known growth factors and cytokines than to PTH [3].

Based on the role of the RAS as crucial pathway involved in pathogenesis and progression of DN, along with the previous studies demonstrating interaction between PTHrP and AngII in the kidney [2, 4], heart [5] and in vascular smooth muscle cells [6,7] the current study aimed at evaluating the effect Ang II antagonists in an experimental model of DN using one pharmacological drug; losartan as an angiotensin receptor blocker (ARB) and a non-pharmacological agent nitro-oleic acid (Olive oil) guided by the recent recommendations provided by American diabetic association (ADA) regarding the value of ARBs in DN [1].

Losartan is a reversible, competitive inhibitor of the AT1 receptor (AT1R) while nitro oleic acid (NO2-OA) binds directly to the AT1R without interfering with
angiotensin II binding, suggesting that molecular interactions between NO2-OA and AT1R are likely to differ from ARBs competitive inhibition [8].

The current study was designed to evaluate the possible preventive and therapeutic effects of losartan and NO2-OA on the functional and histopathological parameters of DN and to elucidate the effect of those drugs on PTHrP modulation in an attempt to provide appropriate ground for better management of diabetic nephropathy.

**Materials and Methods**

**Materials**

Chemicals were purchased from Sigma (St. Louis, MO, USA)

1. Streptozotocin powder (STZ), dissolved in fresh 0.05 M citrate buffer, pH= 4.5 and immediately injected intraperitoneally (ip) as recommended by the National Institute of Diabetes Animal Models of Diabetic Complications Consortium.

2. Losartan 10 mg/kg dissolved in 1ml saline in oral gavage [9].

3. 10-Nitro oleic acid was infused at 5mg/kg/day via osmotic minipump [9].

**Experimental animals**

Forty eight adult male Sprague Dawley rats (150-200g) were purchased from the animal care unit. All experimental procedures were carried out according to guidelines for care and use of laboratory animals published by the U.S. National Institute of Health (NIH 1985). The study was approved by Kasr al-ainy Ethical Committee, Faculty of Medicine, Cairo University. Animals had free access to food and water.

**Preparation of diabetic rat**

The animals were injected with streptozotocin (50 mg/kg, i.p.) [10]. Five days after injection, the rats with fasting blood glucose higher than 200 mg/dl were considered diabetic and were used for the experiment which lasted for eight weeks.
Animal groups:

- Control group
- Diabetic nephropathy (DN) group: Diabetic rats that received equal amount of vehicle. The estimated duration for developing nephropathy is 4 weeks after induction of diabetes [11]. Nephropathy was confirmed by significant increase in proteinuria and serum creatinine.
- Pre-DN losartan group (Losartan prophylactic group): received losartan 10 mg/kg dissolved in 1ml saline in oral gavage [9], as prophylaxis to DN, starting from the onset of diabetes, and continued for 4 weeks (the estimated time for occurrence of nephropathy) and then the rats were left without any medications till the end of study.
- Pre-DN NO2-OA (NO2-OA prophylactic group): received NO2-OA infused at 5mg/kg/day via osmotic minipump, as prophylaxis to DN in the same fashion as the losartan prophylactic group.
- Post-DN losartan (Losartan treatment group): received losartan 10 mg/kg dissolved in 1ml saline in oral gavage [9] for 4 weeks, started after the occurrence of diabetic nephropathy till the end of the study.
- Post-DN (NO2-OA)(NO2-OA treatment group): received NO2-OA infused at 5mg/kg/day via osmotic minipump for 4 weeks, started after the occurrence of diabetic nephropathy till the end of the study.

Experimental design

The animals were daily observed for signs of morbidity and mortality. There was no mortality nor changes in food and water consumption and health status in the studied animals. At the end of the study, all rats were subjected to overnight fasting, blood samples were collected from the retro-orbital plexus for assessment of fasting serum glucose level, blood urea nitrogen (BUN), serum angiotensin and plasma creatinine for assessing GFR. Urine samples were collected for measuring creatinine in urine and 24 hour urine were collected, for measuring GFR and urinary albumin excretion (UAE). Systolic blood pressure in rats was measured. Finally, kidney tissue samples were
extracted for histopathological and quantitative real-time PCR gene expressions of parathyroid hormone related protein (PTHrP).

**Measurement of systolic blood pressure**

Using the Power lab data acquisition system (AD instruments; Pty Ltd Australia); an electronic version of the traditional sphygmomanometer cuff method.

**Biochemical assessment**

*Measurement of fasting serum glucose*

The glucose was measured colorimetrically after oxidation of glucose. The absorbance of standard and samples were measured spectrophotometerically against blank at 500nm wavelength.

**Determination of kidney functions**

1. Measurement of UAE using Albuwell M Kit: Murine Microalbuminuria ELISA. Before sampling, rats were deprived from food for 1 day with free access to water.

2. Measurement of serum creatinine and BUN using kits (Bioclin, Santa Coloma, Spain) with absorbance at 340 nm based on the reaction between ammonia and alpha-ketoglutaric acid when urea is hydrolyzed.

3. Determination of GFR was done by calculating the creatinine clearance: 

   \[
   \text{Creatinine clearance} = \frac{\text{urine level of creatinine} \times \text{24hours urine volume}}{\text{plasma level of creatinine} \times 24 \times 60}.
   \]

*Measurement of angiotensin II Enzyme*

Using enzyme Immunoassay (EIA) Kit (RayBio®).

*Quantitative analysis of gene expression of PTHrP by real time PCR*

Total RNA was extracted from kidney tissue homogenate using Qiagen tissue extraction kit (Qiagen, USA) according to manufacturer’s instruction. Complementary DNA was synthesized from 1 μg RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer’s protocol (#K1621, Fermentas, Waltham, MA, USA).
Real-time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction contained gene-specific primer pairs for PTHrP with following sequence Sense 5'-TGGTGGTTCCTGCTCAGCTA-3', antisense 5'-CCTCGTCGTCTGACCCAAA-3' and beta actin sens :5’-CAG GAT GGC GTG AGG GAG AGC-3’,antisense:5’- AAG GTG TGA TGG TGG GAA TGG-3’. Relative expression of gene mRNA was calculated. All values were normalized to β-actin which was used as the control housekeeping gene and reported as fold change over background levels.

**Histopathological examination**

Kidneys from all groups were dissected and fixed immediately in 10% formol saline. Then the specimens were washed, dehydrated in ascending grades of ethanol, cleared in xylene and impregnated in paraffin. Sections of 5μm thickness were cut and subjected to hematoxylin and eosin (Hx&E), Masson’s trichrome (MT) and Periodic acid shift (PAS) staining.

**Quantitative morphometric analysis**

All histomorphometric parameters were estimated via the Leica image analyzer software program (Leica Qwin 500C Ltd., Cambridge, England) in ten non-overlapping randomly-selected fields.

1. **For H&E-stained sections:**
   The measured parameters were Bowman's spaces, glomerular areas, tubular diameter, tubular epithelial height and vascular congestion area percent.

2. **For Masson’s Trichrome-stained sections:**
   The area percent of collagen fibers was measured using the measuring field menu; the area and area percentage were masked by blue binary color and estimated using the image analyzer.

3. **For PAS-stained sections:**
   Length of PCT brush border and PAS optical density were assessed.

4. **Histopathology scoring**
Histopathology scoring was carried out using a scoring system reflecting the grading of tubular necrosis and cast formation in H&E stained sections as previously described [12] (0 (none), 1 (<10%), 2 (10–<25%), 3 (25–<45%), 4 (45–<75%), and 5 (>75%)).

**Statistical methods**

Data were analyzed using statistical package SPSS version 24. Data were summarized using mean and standard deviation. Comparisons between groups were done using ANOVA with multiple comparisons Bonferroni post hoc testing. Correlations between variables were done using Pearson correlation coefficients. P-values <0.05 were considered statistically significant.

**Results**

**Changes in fasting serum glucose level**

Induction of diabetes resulted in significant increase in serum glucose level. Serum glucose levels in pre-DN losartan and pre-DN (NO2-OA) groups were significantly reduced compared to diabetic group (GpII). However, prophylactic losartan showed significant improving compared to prophylactic NO2-OA (Fig.1A). Post-DN losartan and post-DN (NO2-OA) group showed significant decline in serum glucose, with no significant difference in relation to each other.

**Results of systolic blood pressure**

SBP level was significantly increased in diabetic rats. Prophylactic losartan and NO2-OA resulted in significant improvement of SBP, with much more improvement with losartan (fig.1B). Treatment of diabetic rats with either losartan or NO2-OA showed significant reduction in SBP.

**Results of kidney function parameters: UEA, GFR, urea**

Kidney function parameters were significantly deteriorated in diabetic rats relative to control rats with increase in urea and UAE and decrease in GFR (fig.1C-E). Prophylactic losartan showed partial protection from DN presented by significant decline in urea and UAE and significant rise to GFR. Similarly, prophylactic NO2-OA
showed significant improvement in these parameters. Losartan treatment showed significant lower urea and UAE and higher GFR.

**Changes in serum angiotensin and PTHrP expression in renal tissue**

Serum angiotensin and PTHrP expression showed a significant increase in diabetic rats (fig.1F). After prophylactic administration of both losartan and NO2-OA, serum angiotensin level decreased significantly and PTHrP expression recovered significantly.

Regarding the treatable effect of both drugs, the reduced levels of serum angiotensin and PTHrP expression were significantly improved compared to diabetic rats. Only angiotensin level showed significant difference in post-DN losartan relative to post-DN NO2-OA group with non-significant increase in PTHrP. Correlation studies showed that PTHrP showed a strong positive correlation with angiotensin, urea and UAE and negative correlation with GFR as shown in figure 5.

**Histopathological Results**

**Examination of H&E stained sections (fig1 H,I, fig 2).**

Examination of the DN group revealed pleopathological glomerular and tubular changes with statistically significant deterioration in tubular necrosis and cast formation. Comparing the protective role of losartan and NO2-OA demonstrated that pre-DN losartan resulted in preservation of glomerular and tubular architectural patterns while NO2-OA showed only partial conservation. Losartan or NO2-OA treatment after DN slightly improved glomerulosclerosis.

**Assessment of vasculopathy in H&E stained sections (Fig 2)**

The DN group exhibited severely dilated congested BV surrounded by hyalinosis. After prophylactic administration of either losartan or NO2-OA, marked statistically significant improvement in vascular congestion occurred with only few congested blood capillaries and minimal extravasation still noticed in the pre-DN NO2-OA group. Vascular congestive features on the other hand showed only minimal amelioration in both post DN treated losartan and NO2- OA groups.
Examination of PAS stained sections (Fig 3)

Diabetic rats displayed features of glomerulosclerosis manifested by excessive mesangial matrix deposition causing obliteration of the capillary lumina. Glomerular tuft adhesion, Bowman’s capsule thickening and loss of the apical brush border of renal tubules were also predominant features in the DN group. Prophylactic losartan resulted in preservation of the tubular brush border with normal tubular basal laminae, while prophylactic NO2-OA showed only partial protection with areas of normal PAS reaction and others of focal thickening. Administration of either losartan or NO2-OA after induction of DN showed persistence of Bowman’s capsule and capillary tuft basement membrane thickening in the losartan group and failure to restore the degenerated tubular brush border in the NO2-OA group.

Examination of MT stained sections (Fig 4)

The DN group displayed features of diabetic glomerulosclerosis and tubulointerstitial fibrosis. The pre-DN losartan group showed normal collagen distribution pattern around renal tubules and in the glomeruli except for localized areas of tuft-to-capsule adhesions, while pre-DN NO2-OA group showed partial conservation of the capillary lumina with occasional focal collagen deposition. Losartan treatment resulted in recovery from the glomerular sclerotic pattern but with compensatory tuft hypertrophy while treatment with NO2-OA resulted in non-significant improvement in glomerulosclerosis.

Histomorphometric parameters (Table 1)

Assessment of glomerular parameters showed significant improvement with prophylactic losartan and NO2-OA while the treatment groups on the other hand showed non-significant differences relative to the diabetic group. Regarding tubular parameters, the tubular diameter and tubular epithelial height showed significant increase in pre-DN losartan, pre-DN NO2-OA, and post-DN losartan groups, while the bush border length demonstrated significant increase only with pre-DN losartan.
Discussion

This study has been designed to compare the possible preventive and therapeutic effect of losartan and NO2-OA on DN, and to evaluate their effects on PTHrP modulation in the kidney of diabetic rats.

The DN group in the current study demonstrated various functional changes in the form of deterioration of renal functions and significant elevation of SBP which proved the development of hypertension with DN. This latter finding coincides with the previous reports of increased incidence of hypertension in patients of DN [13]. Systemic hypertension has been also previously reported to cause intraglomerular hypertension leading to glomerular hypertrophy and injury [14]. Marked obliteration of the glomerular capillary tuft secondary to excessive collagen and mesangial matrix deposition demonstrated in our study readily suggests the occurrence of glomerular hypertension in DN group.

PTHrP expression in renal tissues and serum angiotensin were also significantly higher in the DN group in the current study. This adds further evidence to the previous reports demonstrating the involvement of PTHrP overexpression in renal injury [15]. Our work also agrees with previous studies that suggested the involvement of PTHrP in the mechanisms related to Ang II-induced renal injury [16,17]. Furthermore, exogenously administrated Ang II, by its AT1 receptor, increases PTHrP expression in glomerular and tubular cells as well as in vascular smooth cells both in vivo and in vitro. Interestingly, PTHrP in the current study showed a strong positive correlation with angiotensin, urea and UAE and negative correlation with GFR. These results are concordant with the previous studies reporting overexpression of PTHrP in the renal tubules and glomeruli in diabetic mice, which was associated significantly with increased UAE [18]. The authors also reported increase in the risk of developing proteinuria in mice with higher PTHrP levels. This also agrees with our results that PTHrP was involved in renal injury associated with DN.

The current study evaluated the possible preventive and therapeutic effects of losartan and NO2-OA on the functional and histopathological parameters of DN.
Administration of losartan as ARB either pre- or post-DN showed significant improvement in SBP and fasting serum glucose level compared to diabetic group, with more improvement in pre-DN group. Concordant results have been previously reported [19]. This hypoglycemic effect of losartan could be due to the effect of losartan in increasing insulin sensitivity and β-cell responsiveness to glucose and also through enhancement of glucose homeostasis through several insulin signaling pathways [20]. This effect was suggested to occur at the levels of both tyrosine phosphorylation and phosphatidylinositol 3-kinase activation as well as mitogen-activated protein kinase [21].

Our results also showed that pre-DN losartan significantly improved kidney function and was even more effective than post-DN losartan regarding urea, UAE and GFR with a significant difference concerning UAE. These observations are concordant with the previous studies reporting reduction in progression of DN with losartan [22]. Our study has provided an in-depth histopathological analysis of these observations demonstrating increment in tubular epithelial height, improvement in tubular lining with predominance of vesicular nuclei with prominent nucleoli and significant reduction in glomerular and tubular injury markers. Taken together, these data along with the previous histopathological reports [23] provide considerable support for the view that the hallmark features of renal damage including glomerulosclerosis and renal tubular necrosis occur as a result of intense intrarenal vasoconstriction subsequent to renin–angiotensin activation [24, 25].

The renoprotective effect of losartan as an ARBs may be due to BP reduction and increase in renal blood flow with reduction in intra-glomerular pressure [26]. This was evidenced in the current study by conservation of the glomerular capillary lumina, reversion of the glomerular sclerosis pattern, recession of mesangial proliferation and improvement of vaculopathy in losartan-treated groups relative to their diabetic counterparts.

In the current study, administration of losartan resulted in significant decrease in PTHrP levels. These results supports the view that Ang II-induced renal damage is
associated with renal PTHrP upregulation [15] and that Ang II increases PTHrP expression in glomerular and tubular cells both in vivo and in vitro [3].

NO2-OA administration showed a significant decline in fasting serum glucose level in both pre- and post-DN groups relative to DN group, with more improvement in pre-DN group. Systolic blood pressure was also improved significantly after pre- and post-DN NO2-OA administration with non-significant difference between both groups. Our data showed improvement in kidney function and glomerular damage parameters only in pre-DN NO2-OA rats versus diabetic rats with non-significant effect with post-DN NO2-OA rats. This could be explained by the actual occurrence of DN pathological changes interfering with maximum beneficial effect of the drug.

NO2-OA has been previously reported to induce non-significant reduction in diabetic glomerulosclerosis [9]. This adds further support to lack of complete resolution of the sclerotic pattern observed in the glomeruli of DN group following NO2-OA administration in our study.

There is a lack of studies concerning the relation of NO2-OA with PTHrP. This work showed a significant reduction in both serum angiotensin and PTHrP after NO2-OA administration with a more significant decline in pre-DN group. This effect may be due to reduction of AT1R mRNA level and gene expression with subsequent angiotensin reduction [27] or due to binding to AT1R, which further reduces heterotrimeric G-protein coupling, and inhibits IP3 (inositol-1,4,5- triphosphate) and calcium mobilization, without inhibiting Ang II binding to the receptor [8].

The current study offers losartan and nitro-oleic acid as promising lines for enhancing renal sparing and maintaining functional integrity of the kidneys in the diabetic patients. The two drugs, especially losartan, can be suggested as prophylactic agents to prevent the deleterious effect of diabetic nephropathy and its undesired consequences of glomerulosclerosis and tubular injury.

Although the results of the current study suggest the superiority of losartan over nitro-oleic acid in ameliorating diabetic nephropathy, yet, the current study used a rat
model, and dissimilar outcome in humans could be a possibility. Thus, generalizations cannot be made. Also, in a future study, additional testing with larger sample sizes and/or changes to the dosage of losartan and nitro-oleic acid could more precisely determine the significant difference between the studied groups, especially in the post-diabetic nephropathy groups.

Conclusions

Both losartan and NO2-OA were found to improve DN. This study postulated that there is no difference in the preventive effect of both drugs in improving hyperglycemia and elevated SBP as well as in improving kidney function and tubular injury. However, therapeutic effect of losartan on DN may be more beneficial than NO2-OA effect especially in alleviating diabetes-induced vasculopathy and glomerulosclerosis. Moreover, our data highlight the potential role of Ang II antagonist in protection and regression of DN and reduction in PTHrP overexpression supporting the previously reported role of Ang II in regulating PTHrP expression.

References


FIGURE LEGENDS

Figure 1. A) Fasting serum glucose, B) Systolic blood pressure (SBP), C) Albumin in urine (UAE), D) Glomerular filtration rate (GFR), E) Urea, F) Serum angiotensin, G) PTHrP expression in renal tissue among studied groups. H) Tubular necrosis score, I) Tubular cast score. Each vertical bar represents the mean ± SD of 10 animals.
* : statistically significant compared to control group,
# : statistically significant compared to DN group,
$ : statistically significant compared to pre-DN losartan group
@ : statistically significant compared to pre-DN (NO2-OA) group
& : statistically significant compared to post-DN losartan group

Figure 2. (A) Control group showing normal renal corpuscle composed of a normal glomerulus (G) formed of capillary tuft surrounded by Bowman’s space (S). MD: macula densa, P: PCT, D: DCT. (B) DN group showing mesangial matrix deposition (Mx), extraglomerular mesangial expansion (arrow head), congestion of glomerular capillaries with occasional aneurysmal dilatation (arrows) and widening of urinary space (S) secondary to
capillary tuft retraction. Some tubules show intraluminal exfoliation of epithelial cells (curved arrows); others appear obliterated (O) with casts. (C) Pre-DN losartan group showing preservation of the glomerular (G) architectural patterns. Most of the tubules (T) appear healthy with vesicular nuclei and prominent nucleoli (arrows). (D) Pre-DN No2 group showing glomerulus (G) with moderately increased mesangial matrix. Some tubules (T) are degenerated, others appear normal (N). (E) Post-DN losartan group showing glomerulus with tuft–to-capsule adhesion (Ad) (synechiae) and slight increase in mesangial matrix (Mx). Normal macula densa (MD) is observed. (F) Post-DN NO2-OA group showing glomerulus (G) with mesangial hypercellularity. Few tubules (T) appear filled with casts. (Hx&E x400) (Scale bar 50μm). (G,H,I,J,K,L,M showing vascular changes) (G) Control group showing normal vascular pattern (H) DN group showing severely dilated congested BV surrounded by hyalinosis. (I) Pre-DN losartan group showing obvious improvement of vascular congestion. (J) Pre-DN NO2-OA group showing few congested blood vessels in addition to blood extravasation (E). (K) Post-DN losartan group showing markedly dilated congested BV. (L) Post-DN NO2-OA group displaying obviously dilated congested BV in close vicinity of a glomerulus and surrounded by extra-glomerular mesangial cell expansion. (M) Graph showing area percent of vascular congestion in different groups; data are presented as mean±SD (p<0.05 in DN vs control, Pre-DN losartan vs DN and Pre-DN NO2-OA vs DN) (*: statistically significant compared to control,#: statistically significant compared to DN) (G,H: x100, Scale bar 200μm; I,J,K: x400, Scale bar 50μm, L: x200, Scale bar 50μm).

Figure 3. PAS stained sections of: (A) Control group showing positive PAS reaction in the intact apical brush border (arrow heads) and basement membrane of the renal tubules (thin arrows) and capillary basement membrane (thick arrows). (B) DN group showing excessive mesangial matrix (Mx) deposition (Early stages of Kimmelstiel-Wilson lesion) causing obliteration of the capillary lumina and tuft adhesion (Ad) to Bowman’s capsule. Areas of focal thickening of the parietal layer of Bowman’s capsule (B), loss of the apical brush border of renal tubules (T), thickening of the basal membrane of macula densa (curved arrow) and walls of blood vessels (arrow heads) and a degenerated glomerulus (G). (C) Diabetic rats adrenal medulla displaying thickening of the basement membrane
(arrows) of collecting tubules (C), loop of Henle (H) and blood vessels (BV).  

**D** Pre-DN losartan group showing restoration of tubular brush border (arrow heads) with normal thin tubular basal laminae (arrows).  

**E** Pre-DN NO2-OA group showing glomeruli with areas of normal PAS reaction (arrow heads), others with localized thickening (curved arrows).  

The Bowman’s capsule shows few areas of focal thickening (thick arrow) and others of normal thickness (thin arrow).  

**F** Post-DN losartan group showing thickened Bowman’s capsule (curved arrow) and glomeruli with thickened capillary tuft basement membranes (arrow heads) with tuft-to-capsule adhesions (Ad).  

**G** Post-DN NO2-OA group showing hypertrophied glomerulus (G) almost filling the Bowman’s space and surrounded by thin corrugated Bowman’s capsule (arrow). Most of the tubules exhibit degenerated brush border.  

**H** Graph showing optical density of PAS in different groups; data are presented as mean±SD (p< 0.05 in DN vs control, Pre-DN losartan vs DN, Pre-DN NO2-OA vs DN, Post-DN losartan vs DN and Post-DN NO2-OA vs DN) (*: statistically significant compared to control, #: statistically significant compared to DN)  

*(PAS x400) (Scale bar 50μm)*

**Figure 4:**  

(A) Control group demonstrating normal collagen distribution  

(B) DN group displaying sclerosed glomeruli with extensive collagen deposition (arrows) causing marked implosion of the capillary loops. Some glomeruli appear severely collapsed (C); others show tuft-to-capsule adhesion (arrow heads).  

(C) DN group showing tubulointerstitial fibrosis.  

(D) Pre-DN losartan group showing preservation of normal collagen distribution around renal tubules and in the glomeruli except for few localized areas of tuft-to-capsule adhesions (arrow heads). Thin but irregular Bowman’s capsule (B) is observed.  

(E) Pre-DN NO2-OA group showing partial conservation of the capillary lumina (arrows) and focal area of increased collagen deposition (arrow head).  

(F) Post-DN losartan group displaying recovery from the glomerular sclerotic pattern but with compensatory tuft hypertrophy and capsule adhesions (arrow head). Collagen bundles in the parietal layer of the Bowman’s capsule show areas of thickening (thick arrow) as well as areas of rarefaction (thin arrow).  

(G) Post-DN NO2-OA group showing few open capillary loops (arrows) in the sclerotic glomeruli and deposition of few thickened collagen bundles in the Bowman’s capsule (arrow head).  

(H) Graph showing area percent
of collagen deposition in different groups; data are presented as mean±SD (p< 0.05 in DN vs control, Pre-DN losartan vs DN, Pre-DN NO2-OA vs DN and Post-DN losartan vs DN) (*: statistically significant compared to control, #: statistically significant compared to DN) (Masson trichrome x400) (Scale bar 50μm)

**Figure 5.** PTHrP correlation studies showing a strong positive correlation with PTHrP and angiotensin, urea and UAE and negative correlation with GFR.

**Table 1.** Histomorphometric parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DN</th>
<th>Pre-DN Losatan</th>
<th>Pre-DN No2-OA</th>
<th>Post-DN Losatan</th>
<th>Post-DN No2-OA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bowman’s space (μm²)</strong></td>
<td>1756.16 ± 41.30</td>
<td>3007.50 ± 15.14</td>
<td>2053.06 ± 66.01</td>
<td>2416.17 ± 25.11</td>
<td>2524.12 ± 81.31</td>
<td>2991.57 ± 39.18</td>
</tr>
<tr>
<td>(p=0.007)**</td>
<td></td>
<td></td>
<td>(p=0.024)*</td>
<td>(p=0.043)*</td>
<td>(p=0.051)NS</td>
<td>(p=1.65)NS</td>
</tr>
<tr>
<td><strong>Glomerular area (μm²)</strong></td>
<td>3123.18 ± 91.43</td>
<td>2001.61 ± 54.02</td>
<td>2769.34 ± 81.15</td>
<td>2309.16 ± 76.44</td>
<td>2187.16 ± 46.02</td>
<td>2052.75 ± 71.09</td>
</tr>
<tr>
<td>(p=0.042)*</td>
<td></td>
<td></td>
<td>(p=0.01)**</td>
<td>(p=0.49)*</td>
<td>(p=0.66)NS</td>
<td>(p=3.02)NS</td>
</tr>
<tr>
<td><strong>Tubular diameter (μm²)</strong></td>
<td>39.87 ± 2.79</td>
<td>22.28 ± 3.1</td>
<td>33.12 ± 4.07</td>
<td>27.63 ± 5.61</td>
<td>29.88 ± 2.09</td>
<td>25.18 ± 1.49</td>
</tr>
<tr>
<td>(p=0.021)*</td>
<td></td>
<td></td>
<td>(p=0.032)*</td>
<td>(p=0.041)*</td>
<td>(p=0.037)*</td>
<td>(p=0.07)NS</td>
</tr>
<tr>
<td><strong>Tubular Epithelial height (μm)</strong></td>
<td>19.72 ± 2.66</td>
<td>8.84 ± 3.19</td>
<td>15.63 ± 2.44</td>
<td>12.02 ± 1.71</td>
<td>11.25 ± 2.13</td>
<td>9.28 ± 1.51</td>
</tr>
<tr>
<td>(p=0.036)*</td>
<td></td>
<td></td>
<td>(p=0.012)*</td>
<td>(p=0.042)*</td>
<td>(p=0.047)*</td>
<td>(p=0.09)NS</td>
</tr>
<tr>
<td><strong>Length of the brush border (μm)</strong></td>
<td>0.82 ± 0.06</td>
<td>0.41 ± 0.01</td>
<td>0.65 ± 0.08</td>
<td>0.50 ± 0.02</td>
<td>0.54 ± 0.07</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>(p=0.041)*</td>
<td></td>
<td></td>
<td>(p=0.04)</td>
<td>(p=0.062)NS</td>
<td>(p=0.058)NS</td>
<td>(p=1.68)NS</td>
</tr>
</tbody>
</table>

*Significant; **Highly significant; NS — non significant

**N.B.** P values for pre-DN Losatan, pre-DN No2-OA, post-DN Losatan and post-DN No2-OA are calculated relative to the DN group.

P values for DN group are calculated relative to the control group

10 randomly chosen, non-overlapping fields (200x) was as follows: 0 (none), 1 (≤10%), 2 (11–25%), 3 (26–45%), 4 (46–75%), and 5 (≥76%) 0 (none), 1 (≤10%), 2 (11–25%), 3 (26–45%), 4 (46–75%), and 5 (≥76%)