Egyptian Journal of Basic and Clinical Pharmacology
December 2013, Vol. 3 No.2: 31-41
http://www.ejbcp.eg.net/

Original Article

Alfacalcidol Enhances the Protective Effects of Lisinopril against Nephrosclerosis in a Rat Model of Hypertensive Nephropathy

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A B S T R A C T

Background and aim: The efficiency of renin-angiotensin system (RAS) blockade against the progression of end stage renal disease is limited because of the compensatory renin rise, the other way round vitamin D suppresses renin biosynthesis. In the present study the effect of low dose alfacalcidol on the progression of renal injury and the impact of its combination with lisinopril in a model of hypertensive nephropathy induced by NG-nitro-L-arginine methyl ester (L-NAME) in rats was investigated.

Methods: Five groups of rats were used: Vehicle control group; L-NAME group (50 mg/kg); L-NAME and lisinopril (10 mg/kg) group; L-NAME and low dose alfacalcidol (0.1ug/kg) group, alfacalcidol and lisinopril group. All substances were given daily for 8 weeks by gavage. Systolic blood pressure (SBP) was monitored at base line and by the end of the 8th week. Thereafter urine and blood samples were collected for renal function, plasma calcium, phosphorus fibroblast growth factor 23 (FGF23) and 1, 25-$(OH)_2$ vitamin D measurements. Kidneys were isolated for histopathological examinations.

Results: Lisinopril significantly reduced elevated SBP, improved renal dysfunction induced by L-NAME compared to alfacalcidol. Alfacalcidol , significantly limited the interstitial fibrosis (15%) as well as glomerulosclerosis (33%) compared with L-NAME and lisinopril treated group despite having lower effect than lisinopril on the elevated SBP induced by L-NAME. Alfacalcidol reduced plasma renin activity (PRA) increased by both L-NAME and lisinopril. Alfacalcidol had no significant effect on plasma FGF23 but lisinopril reduced it. A significant increase in 1,25 -$(OH)_2$ vitamin D level was evident with lisinopril and alfacalcidol (12.6% and 22.43%) compared to L-NAME group.

Conclusion: Alfacalcidol exhibited renoprotective effects by correction of vitamin D deficiency induced by L-NAME and reduction in PRA induced by lisinopril . Combined lisinopril and alfacalcidol treatment had a more protective effect than either agent alone.

Key Words: nephropathy, alfacalcidol , fibroblast growth factor 23, NG-nitro-L-arginine methyl ester , lisinopril, 1,25 -$(OH)_2$ vitamin D

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1. INTRODUCTION

Development of renal sclerotic lesions is one of the most common complications of hypertension **(François et al., 2004)**.The renin-angiotensin system(RAS) plays a pivotal role in many of the pathophysiologic changes that lead to progression of renal disease **(Baltatzi et al., 2011).** Compared with the heart, the efficiency of renin-angiotensin blockade against the progression of end-stage renal disease is limited in humans, suggesting that other additional systems could be involved, at least partly, in this physiopathological process **(François et al., 2004)**.

The major problem of RAS inhibitors, including angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) is the compensatory renin rise due to the disruption of the feedback inhibition of renin production. The increase in renin activity stimulates the conversion of angiotensin I (Ang I) and ultimately angiotensin II (Ang II), which largely limits the efficacy of RAS inhibition. The increased renin can also act through the prorenin/renin receptor, which may cause renal and cardiovascular damages independent of Ang II **(Zhang et al., 2008)**.Beside that Ang II molecules, bind to other angiotensin (AT) receptors (eg, AT2, AT3, and AT4) that are not blocked by ARBs. AT3 or AT4 have unknown effects and although AT2 has been reported to have an opposite action to that of

AT1, potentially unfavorable effects such as apoptosis, proinflammatory signal transduction, or chemokine induction have been reported **(Baltatzi et al., 2011).**

1, 25-$(OH)_2$ vitamin D [$1,25(OH)_2D_3$], the hormonal form of vitamin D, is a negative endocrine regulator of the RAS. 1, 25 $(OH)_2$ D_3 suppresses renin biosynthesis **(Katholi and Couri, 2011).** The other way round, RAS activity influences the vitamin D-fibroblast growth factor 23 (FGF-23) - klotho axis. Angiotensin II reduces renal expression of klotho, which, in turn, modulates FGF-23 -signaling and 1-alpha hydroxylase, the enzyme converting calcidiol to calcitriol. Beside its effects on vitamin D metabolism, high levels of FGF-23 or FGF-23 resistance, due to klotho deficiency, are associated with progression of chronic kidney disease (CKD) **(Wolf, 2010).**

In CKD, loss of capacity for excreting phosphate by reduced nephron mass and loss of klotho due to RAS activation and tubulointerstitial damage both further enhance circulating levels of FGF-23 and reduce levels of active vitamin D. As derangement of the vitamin D-FGF-23-klotho axis associates with cardiovascular complications in several studies, the interactions of this axis with the RAS may have therapeutic implications in CKD patients, regarding both renal and cardiovascular outcomes **(de Borst et al., 2011).**

Whether vitamin D or vitamin D analogs have therapeutic effects in intervention or prevention of hypertensive renal injury remains to be tested. Given that vitamin D is able to inhibit renin expression in animals. In addition, targeting klotho deficiency in CKD, by optimizing RAS blockade and correction of vitamin D deficiency, may further reduce cardiovascular disease and progression of kidney injury **(Katholi and Couri, 2011).** We reasoned that combining vitamin D analogs with RAS inhibitors should generate better therapeutic effects. To test this concept, in the current study a rat model of hypertensive nephropathy induced by chronic inhibition of NO synthesis by NG- nitro-L-arginine methyl ester (L-NAME) was treated with a combination of lisinopril and alfacalcidol (one alpha-hydroxyvitamin D_3; a vitamin D analog)..

2. MATERIALS AND METHODS

2.1. Experimental Animals

All animal procedures were carried out in accordance with the National Institute of Health guide for the care and use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics Committee for Ain-Shams University, Faculty of Medicine. Male Wistar rats (weighing 150 to 180 g) purchased from National Research Institute (Cairo, Egypt) were housed at temperature of 22-24°C in individual cages and fed a regular pellet diet *ad libitum*. An adaptation period of 1 week was allowed before initiation of the experimental protocol. A total of 8 weeks of experiments were planned.

2.2. Drugs and Chemicals

Lisinopril was generously provided by Merck, Zenec (USA). Alfacalcidol and NG- nitro- L- arginine methyl ester were purchased from Sigma Chemicals (USA).

2.3. Experimental Protocol

Male Wistar rats (40 rats), were randomly classified into 5 groups (n = 8 in each group). The first group (control) received regular tap water to drink plus 1 ml vehicle (0.1 ml phosphate buffered saline (PBS) containing 0.25% ethanol diluted in water). The second group, (L-NAME) received L-NAME (50 mg/kg/day, dissolved in distilled water) **(Nakamura et al., 1998).** The third group (alfacalcidol) received L-NAME and a low dose alfacalcidol (0.1ug/kg/day, dissolved in 0.1 ml PBS containing 0.25% ethanol)**.** The fourth group (lisinopril) received L-NAME and lisinopril (10 mg/kg/day, dissolved in distilled water) **(Oktem et al., 2011).** The fifth group (combined treated) received L-NAME; alfacalcidol, and lisinopril. All substances were given daily by gavage. The dose of alfacalcidol has been used previously in animals with minimal calcemic effect **(Miyakoshi et al., 2010).**

2.4. Systolic Blood Pressure Measurement

Systolic blood pressure of rats was indirectly measured from the tail of conscious rats at the baseline and at the end of the 8^{th} week by non-invasive blood pressure monitor using the tail cuff technique (ML 125 NIBP, AD Instruments, Australia). The inflated cuff pressure was computed using power lab/85p (ML 785 software program). The average of at least three measurements was taken at each occasion.

2.5. Measurement of Biochemical Parameters

2.5.1. Measurement of Urinary Protein Excretion

The day before sacrifice, animals were transferred to metabolic cages and urine samples were collected for a 24 h period. The amount of urinary protein excretion was measured using the method of **Lowry et al (1951).**

2.5.2. Plasma Parameters

Eight weeks after starting L-NAME, the rats were anesthetized with pentobarbital (50 mg/kg i.p.) **(López-Talavera et al., 1997)**. Blood samples were

collected from the tail vein using ethylenediaminetetraacetic acid (EDTA) as the anticoagulant, and the plasma was stored below -20°C. The kidneys were then rapidly excised from all rats for histological study. Plasma urea nitrogen, creatinine, calcium and phosphate levels were measured using an automatic analyzer (Beckman Instruments GmbH, München, Germany). Plasma 1, $25(OH)_2D_3$ was measured by rat 1,25-$(OH)_2$ vitamin D, ELISA Kit (MyBioSource , San Diego, California, USA). Plasma FGF23 levels were determined using rat fibroblast growth factor 23 ELISA kit (Kamiya biomedical company, USA). Plasma renin concentration (PRC) was defined as the rate of Ang I generation from renin in the sample incubated at pH 6.5 for 90 min with excess exogenous substrate provided from nephrectomized rat plasma **(Varagic et al., 2012).** Ang I generated in the sample was quantified by radioimmunoassay (Diosarin Corp, Stillwater, Minnesota, USA).

2.6. Histopathological Studies

Kidney tissue was embedded in paraffin; 4-6 cortical slices from each kidney were taken, sectioned, and stained with Masson's trichrome reagent. One hundred glomeruli were randomly selected for determination of glomerulosclerosis (Approximately 50 subcapsular and 50 juxtamedullary glomeruli). Glomeruli that exhibited adhesion of the capillary tuft to the Bowman's capsule, capillary obliteration, mesangial expansion, or fibrotic crescents were defined as glomerulosclerotic. The extent of glomerular damage was expressed as the percentage of glomeruli that exhibited sclerosis **(Mizobuchi et al., 2007).** For evaluation of the extent of renal interstitial expansion, the fraction of renal cortex that are occupied by interstitium that stained positively for extracellular matrix components by Masson Trichrome was quantitatively evaluated by a point-counting technique in 10 randomly selected microscopic fields, at a final magnification of×200 under a 100-point grid **(Guo et al., 2001).** Blind analysis was done on all sections by the same observer.

2.7. Statistical Analysis

Statistical analysis was carried out using Graph pad prism, software program, version 5.0 (2007), Inc., CA, USA. All values were expressed as means ± SEM. For all parameters, statistical difference among groups was determined using one way analysis of variance (ANOVA) followed by Benferroni's multiple comparisons test. A P value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Systolic Blood Pressure

After 8 weeks of treatment, systolic blood pressure **(**SBP) significantly increased in the L-NAME group by 70.43 % ($P<0.05$). In the lisinopril and alfacalcidol groups, SBP was lower when compared to L-NAME group by 35.2 % and 25.0% respectively ($P<0.05$). However, in the combined treated group SBP was lower when compared to L-NAME group and lisinopril group by 40.3% and 7.9 % ($P>0.05$) respectively (Figure 1). Two rats died from L-NAME group, one rat from lisinopril group and two rats from alfacalcidol group. No deaths occurred in the combined treated group.

3.2. Biochemical Parameters

3.2. 1. Urinary Protein Excretion and Plasma Parameters

Hypertension induced by NO deficiency was accompanied by significant renal function deterioration. This was indicated by the abnormal increase of urinary protein excretion and elevation of plasma creatinine and blood urea nitrogen concentration in the L-NAME group (4.25, 1.43, and 1.71 fold, of the control values, respectively, $P<0.05$).Treatment with lisinopril was accompanied by an improvement in renal function as indicated by decreased excretion of proteins in urine and the plasma concentration of creatinine and blood urea nitrogen (0.7, 0.37, and 0.29 fold, of the L-NAME values, respectively, $P<0.05$). Moreover, alfacalcidol group showed improvement in renal function as indicated by the decreased excretion of proteins in urine, reduction in the plasma concentration of creatinine and blood urea nitrogen (0.27, 0.3, and 0.23 fold, of the L-NAME values, respectively, $P<0.05$). Combined treatment with both lisinopril and alfacalcidol resulted in significantly ($P<0.05$) lower urinary proteins excretion, plasma creatinine, and blood urea nitrogen levels (75.29, 40.89 and 41.32 fold, of the L-NAME values, respectively) (Figure 2A; B; C and Table 1).

Treatment with lisinopril alone increased plasma calcium (3%), and decreased phosphorus (12.34%) compared with L-NAME treated rats. Treatment with alfacalcidol resulted in higher serum calcium and lower phosphorus levels compared with L-NAME and lisinopril treated rats. Combined treatment with both lisinopril and alfacalcidol resulted in significant increase in plasma calcium, and decrease in plasma phosphorus compared with L-NAME treated group. However, this was not significantly different from alfacalcidol treated group (Figure 2D; E and Table 1).

3.2.2. Plasma FGF23, 1, 25 -dihydroxyvitamin D concentration and renin activity

A significant increase (P<0.05) in the FGF23, plasma renin activity (PRA) and decrease in the 1,25 $(OH)_2D_3$, plasma concentration were found in the L-NAME treated group compared to control group. In the lisinopril treated group, FGF23 concentration significantly (P<0.05) decreased while 1, 25 $(OH)_2D_3$ and PRA increased compared to L-NAME treated group. Alfacalcidol treated group showed insignificant (P>0.05) decrease in the FGF23 concentration and increase in the 1, 25$(OH)_2$ D_3 concentration than L-NAME treated group. PRA decreased significantly in the alfacalcidol treated group compared to lisinopril treated group. Combined treated group showed a more decrease in the FGF23 concentration, PRA and increase in the 1, 25$(OH)_2$ D_3 than either L-NAME; or lisinopril treated groups (Fig. 3, A, B and C).

3.4. Renal Histopathological Changes

Hypertension induced by chronic L-NAME was accompanied by severe sclerotic lesions in the glomeruli (Fig 4 A) shown by varying degrees of glomerular sclerosis, affecting 60 % of glomeruli (P<0.05 versus normal control). An increase in interstitial area (30%) was also observed in L-NAME treated rats (P < 0.05 versus normal control) (Fig 4 B). Lisinopril produced a significant decrease in the percentage of glomeruli that exhibited sclerotic changes (40%) and significantly limited the increase in interstitial area (23%) compared with L-NAME group (P<0.05). Alfacalcidol produced a significant decrease in the percentage of glomeruli that exhibited sclerotic changes (33%) and significantly limited the increase in interstitial area (15%) compared with L-NAME group (P<0.05). Lisinopril when combined with alfacalcidol produced a further improvement (22% and 12% for glomeruli that exhibited sclerotic changes and interstitial area respectively; P< 0.05versus L-NAME) (Fig. 5 A &B).

Table 1. Effect of lisinopril (10 mg/kg/day by gavage) and alfacalcidol (0.1ug/kg/day by gavage) either alone or in combination on L-NAME-induced changes in renal functions, plasma calcium and phosphorus levels in rats at the end of the 8th week.

Parameters	Control group(n=8)	L-NAME group(n=6)	L-NAME - lisinopril group(n=7)	L-NAME - alfacalcidol group (n=7)	L-NAME - lisinopril alfacalcidol - group(n=8)
Urinary protein excretion (mg/day)	18.88± 0.72	99.17*±2.4	29.71#$± 1.49	72.29#± 0.99	24.50#$± 0.68
Creatinine (mg/dl)	0.61 ± 0.03	1.48*± 0.06	0.93#±0.04	1.03# ±0.04	0.86# ±0.05
Blood urea nitrogen (mg/dl)	19.50±0.96	52.83*±0.95	37.50#±0.76	40.67#±1.45	31.0#$±1.59
Ca(mg/dl)	9.78±0.05	9.30*± 0.04	9.59#$±0.05	9.90#± 0.06	9.86#±0.01
P (mg/dl)	3.96±0.05	5.59*±0.08	4.90#$ ±0.08	4.65#±0.08	4.50#± 0.05

Five groups were used, Control (received vehicle) group; L-NAME (50 mg/kg/day) group (n=6); L-NAME + lisinopril group (n=7); L-NAME + alfacalcidol (n=6); L-NAME +lisinopril +alfacalcidol group(n=8).
Data given are means ±SEM.
Statistical difference among groups was determined using one way analysis of variance (ANOVA) followed by Benferroni's multiple comparisons test
* P<0.05, vs Control group, # P<0.05vs L-NAME group, $P < 0.05 vs L-NAME + alfacalcidol group; by one-way ANOVA.

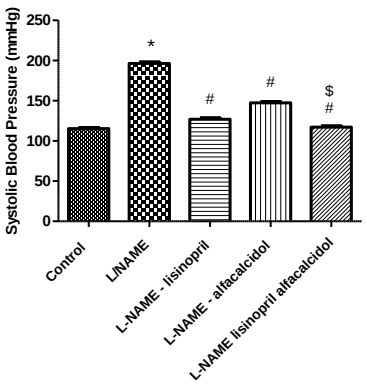


Figure 1: Effect of lisinopril and alfacalcidol either alone or in combination on systolic blood pressure in L- NAME treated rats. Control (vehicle) group (n=8); L-NAME (50 mg/kg) group (n=6); L-NAME + lisinopril(10mg/kg) group (n=7); L-NAME + alfacalcidol (0.1ug/kg) group (n=6); L-NAME + lisinopril +alfacalcidol group(n=8). All substances were given daily by gavage for 8 weeks.

Data given are means ±SEM. Statistical difference among groups was determined using one way analysis of variance (ANOVA) followed by Benferroni's multiple comparisons test

* P<0.05, vs control group, # P<0.05vs L-NAME group, $P < 0.05 vs L-NAME + lisinopril & L-NAME + alfacalcidol group.

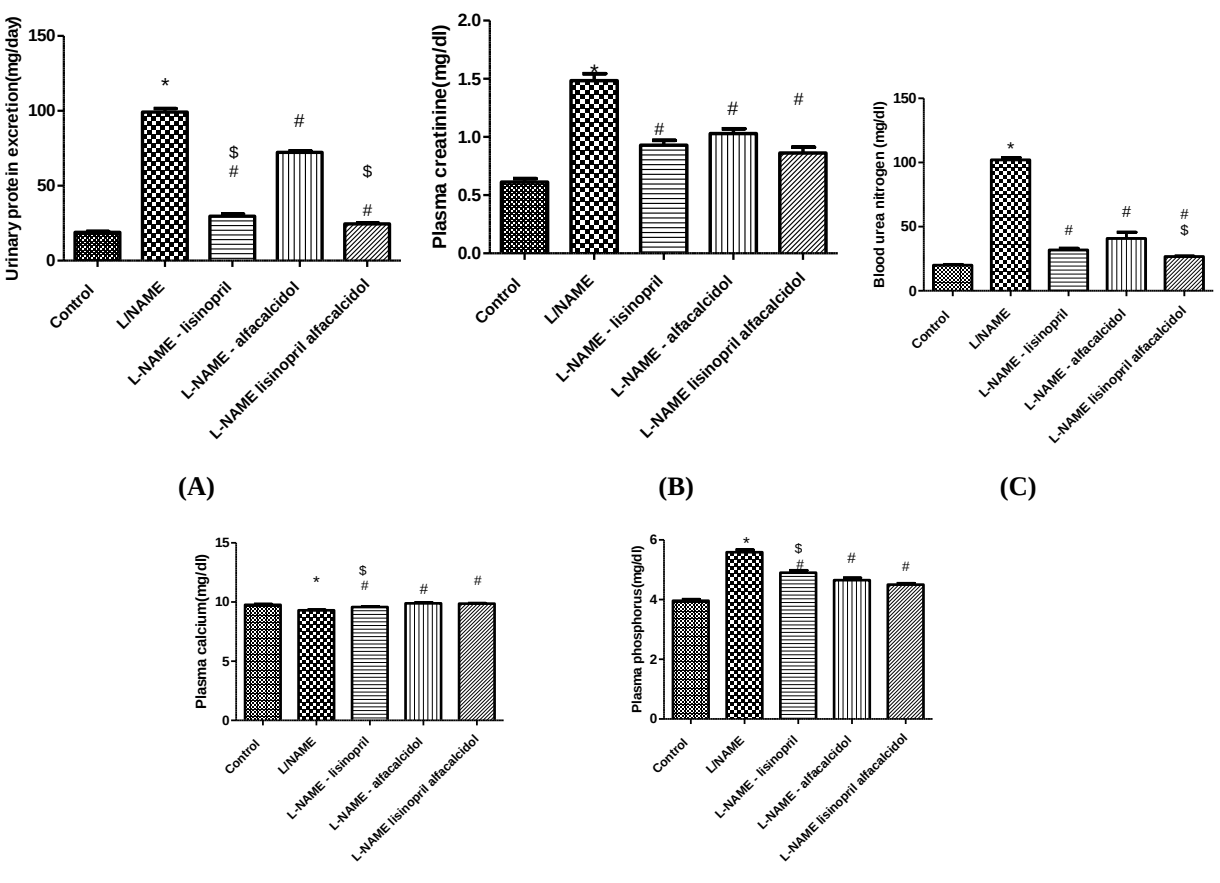
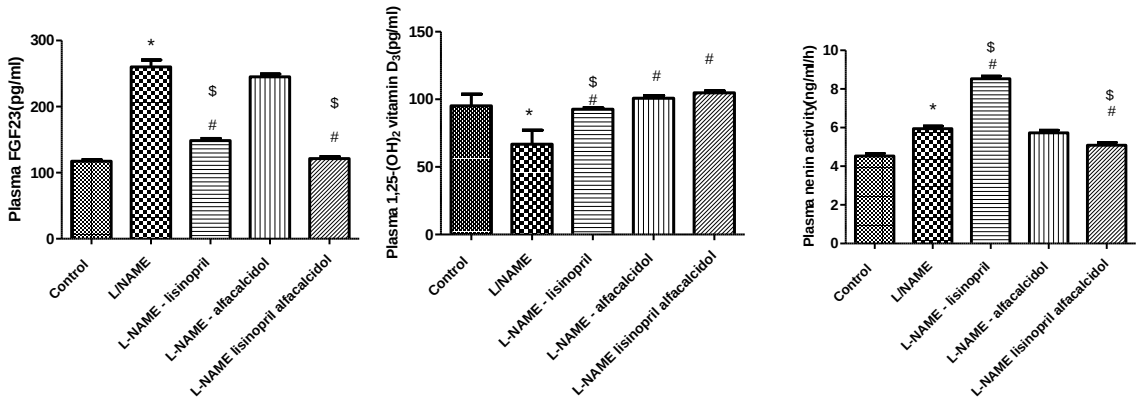


Figure 2: Effect of lisinopril and alfacalcidol either alone or in combination on urinary protein excretion**(A),** plasma creatinine **(B)** , plasma urea nitrogen **(C)** , plasma calcium level **(D)** and plasma phosphorus level **(E)** in L- NAME treated rats. Control (vehicle) group (n=8); L-NAME (50 mg/kg) group (n=6); L-NAME + lisinopril (10mg/kg) group (n=7); L-NAME + alfacalcidol (0.1ug/kg) group (n=6); L-NAME +lisinopril +alfacalcidol group(n=8). All substances were given daily by gavage for 8 weeks.

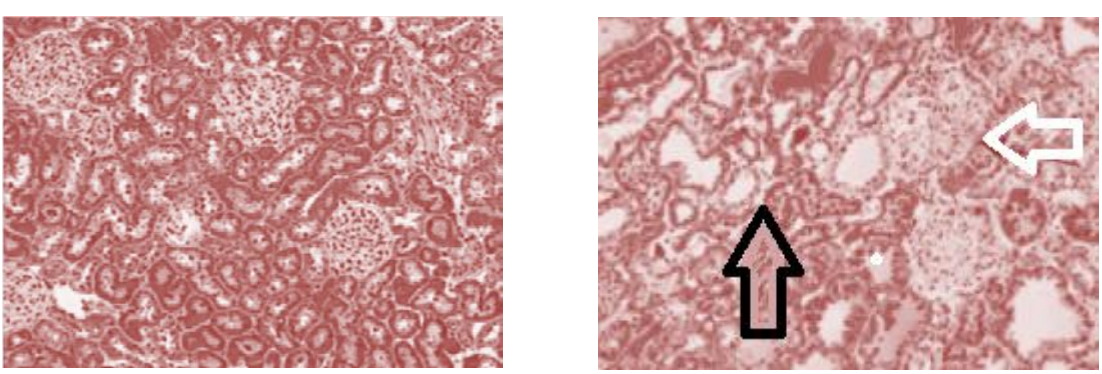
Data given are means ±SEM. Statistical difference among groups was determined using one way analysis of variance (ANOVA) followed by Benferroni's multiple comparisons test.

* P<0.05, vs control group, # P<0.05vs L-NAME group, $P < 0.05 vs L-NAME + alfacalcidol group.



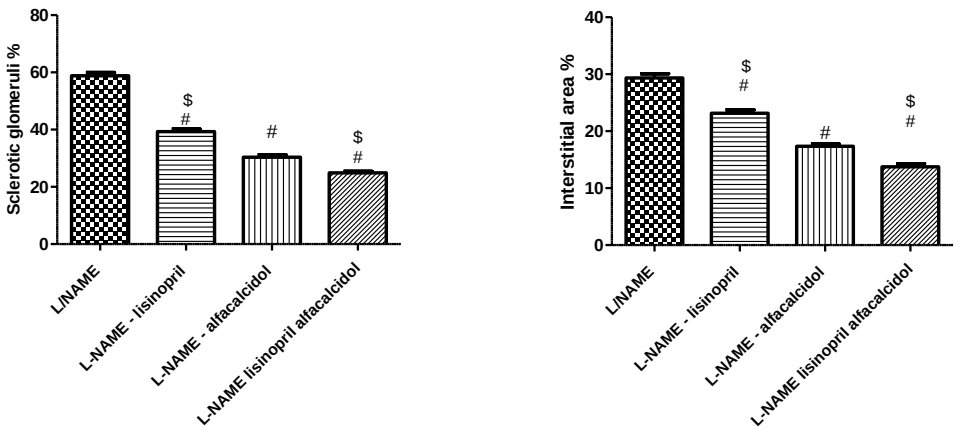
(A) **(B)** **(C)**

Figure 3: Effect of lisinopril and alfacalcidol either alone or in combination on **(A)**, plasma FGF23 (fibroblast growth factor 23) **(B)** plasma 1,25-$(OH)_2$ vitamin D **(C)** plasma renin activity in L- NAME treated rats.. Control (vehicle) group (n=8); L-NAME (50 mg/kg) group (n=6); L-NAME + lisinopril (10mg/kg) group (n=7); L-NAME + alfacalcidol (0.1ug/kg) group (n=6); L-NAME +lisinopril +alfacalcidol group(n=8).All substances were given daily by gavage for 8 weeks.
Data given are means ±SEM. Statistical difference among groups was determined using one way analysis of variance (ANOVA) followed by Benferroni's multiple comparisons test.
* P<0.05, vs control group, #P<0.05vs L-NAME group, $P < 0.05 vs L-NAME + alfacalcidol group.



A **B**

Figure 4: Representative Masson's trichrome stained sections of renal tissue in control rats **(A)** shows intact glomeruli and intact interstitium and in N omega -nitro-L-arginine methyl ester (L-NAME) rats at week 8th **(B)** shows sclerotic glomeruli (white arrow) and expanded interstitial fibrosis(black arrow).Magnification: x200.



(A) **(B)**

Figure 5. Effect of lisinopril and alfacalcidol either alone or in combination on percentage of sclerotic glomeruli **(A),** percentage of interstitial area (**B)** in L- NAME treated rats. Control (vehicle) group (n=8); L-NAME (50 mg/kg) group (n=6); L-NAME + lisinopril(10mg/kg) group (n=7); L-NAME + alfacalcidol (0.1ug/kg) group (n=6); L-NAME + lisinopril +alfacalcidol group (n=8). All substances were given daily by gavage for 8 weeks.

Data given are means ±SEM. Statistical difference among groups was determined using one way analysis of variance (ANOVA) followed by Benferroni's multiple comparisons test

#P<0.05 vs L-NAME group, $P < 0.05 vs L-NAME + alfacalcidol group; by one-way ANOVA. Interstitial area is represented as the fraction of cortical area occupied by interstitium.

4. DISCUSSION

Development of renal sclerotic lesions is one of the most common complications of hypertension **(Weistuch and Dworkin, 1992).** This pathophysiological process is associated with abnormal accumulation of extracellular matrix in renal resistance vessels, glomeruli and interstitium **(Yoshioka et al., 1990).** Studies show that therapeutic interventions such as the blockade of the RAS slows the progression of renal disease in experimental models **(Fujihara et al., 1998)** and human chronic kidney disease (CKD) clinical trials **(Hou et al., 2006).** Although these strategies promote renoprotective effects, they do not halt the progression of renal fibrosis and scarring. Considering that interstitial fibrosis and glomerulosclerosis represent the final common pathway of CKD, therapeutic intervention with drugs that display antifibrotic properties may represent an attractive choice of therapy for arresting the autonomous fibrogenic process in chronic progressive nephropathies. Evidence suggests that the vitamin D receptor (VDR) have a crucial role in the regulation of cell proliferation and differentiation and immunomodulation as well as mineral metabolism **(Mizobuchi et al., 2007).**

The present study demonstrated that chronic L-NAME treated rats developed severe hypertension, deterioration in renal functions, glomerulosclerosis and tubulointerstitial fibrosis. Lisinopril significantly reduced elevated blood pressure induced by chronic L-NAME treatment. Consequently it prevented glomerular capillary pressure elevation which is important for the inhibition of glomerular sclerosis in hypertension. In addition it improved renal function, but did not decrease tubulointerstitial fibrosis to the same extent as alfacalcidol ($p>0.05$).

In contrast alfacalcidol, alleviated interstitial fibrosis as well as glomerulosclerosis in L-NAME treated rats without significant blood pressure reduction than lisinopril treated rats ($p>0.05$). Therefore, alfacalcidol is thought to be effective in mitigating the renal tissue injuries in L-NAME treated rats by a mechanism independent of blood pressure changes. Lisinopril when combined with alfacalcidol produced a further improvement for glomeruli that exhibited sclerotic changes and increase in interstitial volume.

The main mechanism underlying the severe renal fibrosis in chronic L-NAME treated rat appears to be the action of the local RAS in the kidney. Vitamin D receptors (VDR) mediate the action of 1, 25 $(OH)_2$ D_3 to suppress renin and Ang II, **(Yuan et al., 2007)** and VDR inactivation leads to activation of

the RAS and overproduction of Ang II **(Li et al., 2002)**. Ang II is a potent inducer of renal fibrosis **(Wolf, 2006)** and stimulates transforming growth factor beta (TGF-β) production and renal inflammation **(Esteban et al., 2004)**. In the present study, alfacalcidol reduced PRA increased by L-NAME and lisinopril, and this could explain the renoprotective effect of alfacalcidol.

In the present study, lisinopril reduced proteinuria in L-NAME treated rats more prominently than alfacalcidol. This may be a result of more significant blood pressure reduction. Previous study had shown renin-angiotensin system blockade to reduce proteinuria and slow the progression of renal dysfunction in patients with nondiabetic renal disease **(Ishimitsu et al., 2005).** In addition **Oktem et al. (2011)** reported that lisinopril treatment for 6 weeks (10 mg/kg/day) could diminish biochemical alterations in L-NAME induced hypertensive renal damage in Sprague-Dawley rats.

Schwarz et al. **(1998)** reported that calcitriol treatment reduces glomerulosclerosis and albuminuria in subtotally nephrectomized rats. In addition **Makibayashi et al. (2001)** reported that calcitriol attenuates mesangial expansion, glomerulosclerosis, and albuminuria, in rats with anti–Thy-1.1 glomerulonephritis. **Hullett et al. (2005)** reported that a high dosage of calcitriol preserves renal function, in a rat model of chronic allograft nephropathy. **Finch et al (2012)** demonstrated that paricalcitol alone can improve proteinuria, glomerulosclerosis, and interstitial infiltration and reduce renal oxidative stress in uremic rats.

The combined group in this study was given lisinopril and alfacalcidol and showed more blood pressure reduction than either agent alone. In addition, the renal dysfunction, proteinuria, and glomerular injuries were improved comparably to the lisinopril group. Thus, the simultaneous blockades of Ang II and alfacalcidol may be an effective strategy for the prevention of hypertensive renal injuries. **Finch et al (2012)** reported that the effects of paricalcitol may be amplified when an ACE inhibitor is added since cotreatment with both compounds seems to have an additive effect on ameliorating uremia-induced changes in inducible nitric oxide synthase (iNOS) and Cu/Zn superoxide dismutase (Cu/Zn-SOD) expression, peroxidase activity, and renal histomorphometry.

Alfacalcidol treatment, however, can lead to hypercalcemia and hyperphosphatemia, increasing the risk for the progression of kidney disease. In this study, we used a low dose alfacalcidol with lower calcemic and phosphatemic effects. We showed that treatment with alfacalcidol alone produced a significant renoprotective effect in hypertensive nephrosclerosis in rats, and that the effect occurred without significant affection of plasma calcium or phosphorus.

FGF23 is a bone-derived hormone that regulates phosphate and vitamin D metabolism through fibroblast growth factor receptor (FGFR) /α-Klotho co-receptors **(Martin et al., 2012)** that are expressed in a limited number of tissues, including the kidney **(Kurosu et al., 2006).** In the kidney, FGF23 suppresses sodium-phosphate co-transporter function leading to phosphaturia and reduces 1, $25(OH)_2$ D_3 synthesis in the proximal tubule **(Li et al., 2007).** Physiologically, FGF23 is part of a bone-kidney feedback loop, where circulating 1, 25 $(OH)_2$ D_3 stimulates FGF23 production in bone and FGF23 suppresses $1,25(OH)_2D_3$ production in the kidney **(Liu et al., 2006)**.

Elevations of circulating FGF23 also occur early in the course of CKD, where it stimulates phosphaturia to maintain phosphate balance and contributes to the development of secondary hyperparathyroidism through suppression of $1,25(OH)_2$ D_3 levels **(Hasegawa et al., 2010)**. In chronic kidney disease, FGF23 is one of the strongest predictors of mortality **(Stubbs et al., 2009)**. In addition, elevated circulating FGF23 concentrations are independently associated with more rapid progression of kidney disease **(Fliser et al., 2007).**

Angiotensin converting enzyme 2 (ACE2) was found to be reduced by excess FGF23 **(Dai et al., 2012).** ACE2 is a negative regulator of the RAS that has vasodilator and natriuretic effects, leading to reduced blood pressure **(Gurley et al., 2006)**. A direct effect of FGF23 to suppress ACE2 provides an alternative explanation for the recently proposed associations between vitamin D deficiency, activation of the RAS and regulation of α-Klotho expression **(de Borst et al., 2011)**.

FGF23 regulates several genes associated with renal injury. These include Cfi, which has been shown to contribute to inflammatory and acute renal injury **(Chan et al., 2009)** and carbonic anhydrase 14 (car14), whose inactivation in transgenic mice leads to progressive renal injury **(Datta et al., 2010)**. Finally, pathway analysis identified TGF-β and tumor necrosis factor-alpha (TNF- α) signaling pathways as being involved in FGF23 responses in the kidney. The TGF-β pathway is amplified in most forms of CKD in humans and experimental animals. This will eventually control fibrogenesis, apoptosis, epithelial-to-mesenchymal transition, and inflammation leading

to glomerulosclerosis and tubulointerstitial fibrosis **(Ju et al., 2009)**.

Tumor necrosis factor alpha (TNF – α) is a central proinflammatory agonist mediator that might contribute to renal disease progression and cardiovascular events **(Knight et al., 2004)**, and even in non-calcified aortas in patients with CKD an increased TNF immunoreactivity is displayed **(Koleganova et al., 2009)**. It was also found that FGF23 associated increases in vascular cell adhesion molecule 1 (VCAM1), which is expressed in proximal tubule cells in response to inflammatory renal diseases **(Tu et al., 2001)**, and interferon-induced guanylate-binding protein 2 (GBP2), which regulates cell growth and matrix metalloproteinase expression **(Kresse et al., 2008)**.

Nevertheless, despite the known action of active vitamin D therapies to increase FGF-23, this should probably still form an important part of the management of patients with advanced renal disease at low doses **(Fish and Cunningham, 2012).** In the present study, in hypertensive nephrosclerotic rats, alfacalcidol did not increase FGF-23 and normalized the low level of 1,25 $(OH)_2D_3$. Lisinopril alone, or in combination with alfacalcidol, further decreased FGF23. Cotreatment with lisinopril and alfacalcidol had an additive effect in increasing 1,25 $(OH)_2$ D3.

5. CONCLUSION

The present study demonstrated that alfacalcidol can suppress the progression of renal injury via normalization of 1, 25 $(OH)_2$ D_3 and this effect is amplified when blood pressure is controlled via RAS blockade. In addition RAS blockade reduces FGF23 and modulates signaling pathway that was found to be independently associated with faster progression of CKD. Combination therapy with ACEI and alfacalcidol may represent a novel and beneficial therapeutic strategy for arresting the progression of CKD.

6. ACKNOWLEDGEMENT

The author wishes to thank Dr. Naglaa S.Ahmed, Professor of Pathology, Faculty of Medicine, Ain Shams University for her help and support in histopathological part of this study.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this work.

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