The mechanism of attenuation of epithelial-mesenchymal transition by a phosphodiesterase 5 inhibitor via renal klotho expression

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The mechanism of attenuation of epithelial-mesenchymal transition by a phosphodiesterase 5 inhibitor via renal klotho expression

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모교에 첫 발을 내딛은지 어느덧 20 여년이 훌쩍 지났습니다. 저를 큰 사람으로 만들어 준 소중한 모교를 영원히 기억하며 항상 보답하는 마음으로 의학도의 길을 걸어나갈 것을 다짐합니다.

2017 년 12 월 저자 드림

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ABSTRACT

The mechanism of attenuation of epithelial-mesenchymal transition by a phosphodiesterase 5 inhibitor via renal klotho expression

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Background: Phosphodiesterase-5 (PDE-5) inhibitors induces vasodilation in several organs by blocking cyclic guanosine monophosphate (cGMP) degradation. However, the existence of alternative mechanism of action in case of an impaired nitric oxide (NO) system remains controversial. Previous studies suggested that decreased NO bioavailability may result in

the down-regulation of klotho expression, but the relationship between klotho and NO remains obscure. Therefore, we investigated whether a PDE-5 inhibitor could preserve epithelial mesenchymal transition (EMT) and relationship exists between the NO and renal klotho expression.

Methods: Ten weeks Sprague-Dawley (SD) rats (N=24, 200 g, male) were divided (N=6) into four groups, which received: A low salt diet (LSD), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) 1 mg/mL in drinking water, udenafil 5 mg/kg subcutaneously and both for 4 weeks. Urine nitrate/nitrite, Neutrophil gelatinase-associated lipocalin (NGAL), and cGMP were measured using enzyme-linked immunosorbent assay (ELISA). Kidney was subjected to evaluate proliferative cell nuclear antigen (PCNA), alpha-smooth muscle cell antigen (α-SMA), E-cadherin, and klotho expression.

Results: Urine cGMP decreased after treatment of PDE-5 inhibitor compared with control due to blocking degradation of cGMP (p<0.05, control vs Udenafil and L-NAME with Udenafil groups). Urine NGAL increased after treating of L-NAME and attenuated after using PDE-5 inhibitor (p<0.05, control vs L-NAME and L-NAME with Udenafil). PCNA and α-SMA (EMT markers) increased after L-NAME treatment and normalized after using PDE-5 inhibitor. E-cadherin (anti-EMT marker) result was the opposite.

Klotho expression showed trend to increase in the L-NAME with PDE-5 inhibitor group compared with the L-NAME group, however, eNOS expression did not change after treatment of L-NAME or PDE-5 inhibitor compared with control.

Conclusion: PDE-5 inhibitor alleviates EMT in the kidney via klotho modulation independent of the NO system.

Key Words: phosphodiesterase-5 inhibitor, klotho, epithelial–mesenchymal transition, nitric oxide system

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I. INTRODUCTION

Inhibition of cyclic guanosine monophosphate (cGMP) degradation

by phosphodiesterase-5 (PDE-5) targeted compounds has proven most successful in the treatment of pulmonary arterial hypertension (PAH) to date, although PDE-5 inhibitors are usually used to treat erectile dysfunction (1). However, in kidney diseases, PDE-5 inhibition can reduce albuminuria in subjects with diabetes and ameliorate angiotensin II induced podocyte injury (2, 3). In an animal model of kidney ischemic reperfusion injury, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) showed toxic effect on glomerulus and tubule in several animal models, such as preeclampsia and spontaneous hypertensive rat (4-7). These effects mediated via the modulation of the nitric oxide (NO) system by PDE-5 inhibitors suggest that an increase in end-product in target organ can ameliorate tissue damage in several diseases (7-9). We have previously reported that PDE-5 inhibitors can ameliorate cyclosporine A induced renal injury (10).

The number of studies examining the role of fibroblast growth factor-23 (FGF23) and klotho in chronic kidney disease (CKD) has risen exponentially over the past decade. Tissue levels of the FGF23 coreceptor klotho declines in early CKD and this deficiency is linking to accelerated ageing, cellular senescence, vascular calcification, oxidative stress, and renal fibrosis. At present, methodological difficulties limit the utility of soluble klotho measurements, but animal studies have demonstrated the beneficial

effects of klotho delivery in CKD (11). The in-vivo reduction in endogenous NO production in klotho-deficient mice is reflected by the reduced urinary excretion of NO metabolites and cGMP (an indicator of NO synthesis) (12, 13). Further, in-vitro experimental findings lend support for klotho-induced enhancement of NO production in human endothelial cells via modulation of the activity of endothelial nitric oxide synthase (eNOS), the enzyme responsible for generation of NO (14, 15).

Epithelial-mesenchymal transition (EMT) has been implicated in cancer progression and metastasis, wound healing, and the development of fibrotic disorders, including pulmonary, hepatic, and renal fibrosis (16-18). EMT in the kidney can be induced by CKD-associated renal hypoxia, which is thought to result from a combination of structural and functional changes that include decrease in the number of capillaries, compromised peritubular blood flow resulting from glomerular injury, vasoconstriction due to changes in the levels of vasoactive factors and signaling molecules such as angiotensin II, endothelin, and NO (19).

Therefore, in this study, we investigated whether PDE-5 inhibition could ameliorate EMT via klotho expression in the kidney.

Ⅱ. MATERIALS AND METHODS

1. Animals

The ten-week-old male Sprague-Dawley rats (body weight: 200-250 g) were housed with standard chow and tap water available *ad libitum*. All animal procedures and care protocols were approved by Yonsei University at Wonju Campus Institutional Animal Care and Use Committee.

2. Experimental protocol

2-1. Animal groups and experimental design

The ten-week-old male Sprague-Dawley rats (N=24; body weight 200-250 g) were divided into four groups, which received the following treatments for 4 weeks: a low salt diet (control group; N=6), L-NAME 1 mg/mL in drinking water (L-NAME group; N=6) (47-49), udenafil 5 mg subcutaneously (SQ) (Udenafil group; N=6), and both L-NAME and udenafil (L-NAME and Udenafil group; N=6). The experimental drugs used were L-

NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, Sigma-Aldrich Co., St. Louis, MO, USA) and udenafil (Zydena®, Dong-A Pharmaceutical Co., Seoul, Korea), a PDE-5 inhibitor. Low salt diet can increase sensitivity of renal injury, because its activation of renin-angiotensin system can augment L-NAME induced tubular cell damage. We decided the dosage and duration of L-NAME and udenafil following several other study protocols (7, 47-49). On day 28, blood samples were collected from the jugular vein, and both kidneys were extracted. A portion of each kidney was fixed in 10% neutral formalin and prepared as tissue blocks embedded in paraffin. An additional portion of the kidney was cooled rapidly in liquid nitrogen and stored at -70 °C.

2-2. Biochemical markers

On day 28, serum creatinine was measured using a kinetic colorimetric assay with the Modular equipment (Hitachi High-Technologies Corporation, Tokyo, Japan). Urine nitrate/nitrite and cGMP levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Urine neutrophil gelatinase-associated lipocalin (NGAL) levels were also measured using an ELISA kit

(Abcam, Cambridge, UK)

2-3. Immunohistochemistry

Kidney tissue blocks embedded in paraffin were prepared as 5-μm thick sections, and attached to the silane-coated glass slides. To recover antigenicity, the PT module (Lab Vision, Fremont, CA, USA) was used and heated at 99°C for 15 minutes. For the inactivation of endogenous peroxidase activity, the samples were pretreated with Hydrogen Peroxide Block (Thermo Fisher Scientific, Fremont, CA, USA) for 10 minutes, and washed with Tris-buffered saline plus Tween 20 (TBST buffer; ScyTek Laboratories, Logan, UT, USA). To suppress nonspecific antigens, the samples were treated with Ultra V Block (Thermo Fisher Scientific, Fremont, CA, USA) for 5 minutes followed by incubation with primary antibody for 2 hours. The primary antibodies used were as follows: anti-klotho antibody (Thermo Fisher Scientific, Fremont, CA, USA), anti-eNOS antibody ab66127 (Abcam, Cambridge, UK), anti-PCNA antibody ab9252 (Abcam, Cambridge, UK) diluted 1: 200, 1: 25, and 1:200 respectively. The samples were treated with the horseradish peroxidase (HRP) polymer secondary antibody (Thermo Fisher Scientific, Fremont, CA, USA) for 30 minutes and

then incubated with TBST buffer. Background staining was performed with Mayer's hematoxylin, and then the slices were immersed in distilled water, sealed with Immu-Mount (Thermo Fisher Scientific, Fremont, CA, USA), and examined under a microscope.

2-4. RNA isolation and real-time PCR

The tissues were cut to 0.5 cm pieces, washed twice with phosphate-buffered saline (PBS), and then the RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). The concentration of RNA was measured at 260 nm and 280 nm with a spectrophotometer (Bio-Rad, Hercules, CA, USA). cDNA was synthesized from the isolated RNA by using the Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Less than 1 mg of RNA was mixed with 2 ml 7× gDNA Wipeout buffer and incubated at 42°C for 2 minutes. Next, 1 ml Quantiscript Reverse Transcriptase, 4 ml 5× Quantiscript Reverse Transcription buffer, and 1 ml RT primer mix were added, and incubated at 42 °C for 30 minutes and at 95°C for 3 minutes. The cDNA synthesized in this manner was used as a template for PCR. For the PCR reaction mixture, 400 ng cDNA, 5 ml 2× QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany), 10 pmol

primer, and 30 pmol probe were mixed. The QuantiTect Primer Assay (klotho: QT01570618, Qiagen, Hilden, Germany) was used as the source of the primers. PCR was performed using the RotorGene Real-Time Q-PCR system (Corbett Research, Sydney, Australia). The PCR conditions included the HotStarTaq polymerase activation step; the samples were reacted at 95°C for 15 minutes, denaturation was performed at 94°C for 15 seconds, annealing was performed at 60°C for 1 minute, extension was performed at 72°C for 30 seconds, for a total of 50 cycles. Beta-actin was used as the control. The amount of mRNA obtained was calculated as the relative concentration using the value $2^{-\Delta\Delta Ct}$, and the results were compared.

2-5. Protein preparation and western blotting

Kidney proteins were extracted with 1× cell lysis buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 15,000 rpm at 4°C for 30 min, supernatant was collected and 20 µl of lysate from each sample was run on a 10% dodecyl sulfate (SDS)-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were rinsed in TBST (10 mM Tris-HCL, pH 7.4, 0.9 % NaCl, 0.05% Tween 20, and 1 mM

EDTA) and blocked in blocking buffer (TBST containing 5% bovine serum albumin) for 1 hour at room temperature. PVDF membranes were then incubated with primary antibodies against β-actin, sc-47778 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E-cadherin antibody (Thermo Fisher Scientific, Fremont, CA, USA), and α-SMA antibody ab5694 (Abcam, Cambridge, UK) overnight at 4°C. Subsequently, the membranes were washed, and then incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature. The membrane was developed with enhanced chemiluminescent (ECL) substrate (Li-cor, Lincoln NE, USA) and exposed to UVP chemiluminescence (Biolite LLC, Upland, CA, USA)

2-6. Statistical analysis

The SPSS version 18.0 and Graphpad Prism 5 program was used for statistical comparisons and making graph. The non-parametric *Kruskal-Wallis* and *Mann-Whitney* test were used. A *p* value less than 0.05 was considered significant.

Ⅲ. RESULTS

1. Comparison of the effects of a phosphodiesterase-5 inhibitor on the NO system among the groups

Urine nitrate/nitrite and cGMP levels were measured and normalized to creatinine levels to determine the success of the experimental design. The nitrate/nitrite metabolite levels showed a decreasing trend in the L-NAME and L-NAME with Udenafil group compared to that in the control and Udenafil group. The urine cGMP levels were 2.59±0.88, 1.79±0.99, 1.20±0.22, and 0.69±0.59 nmol/μl for the control, L-NAME, Udenafil and L-NAME with Udenafil group, respectively ($p < 0.05$, control vs Udenafil and L-NAME with Udenafil group) (Fig. 1, 2).

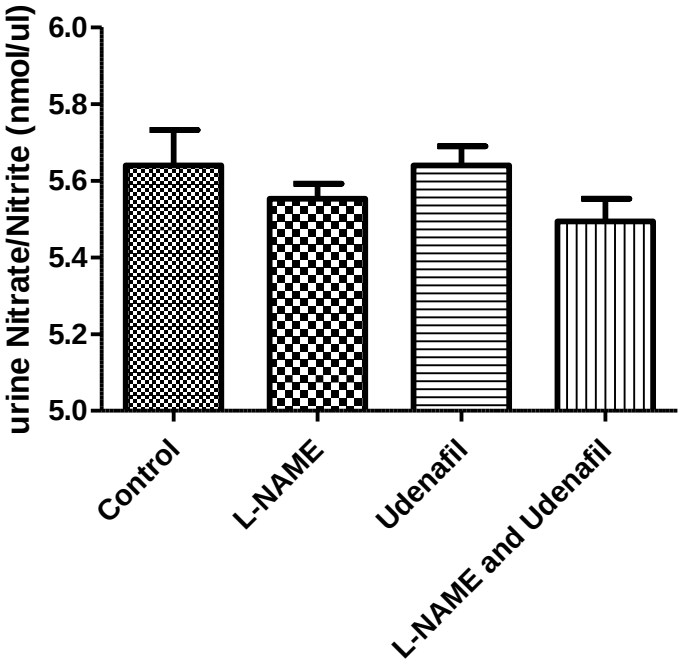


Figure 1. Comparison among the different groups of the effects on the nitric oxide system. Urine nitrate/nitrite metabolite concentration levels showed a decreasing trend in the L-NAME and L-NAME with Udenafil group compared to the control and Udenafil group.

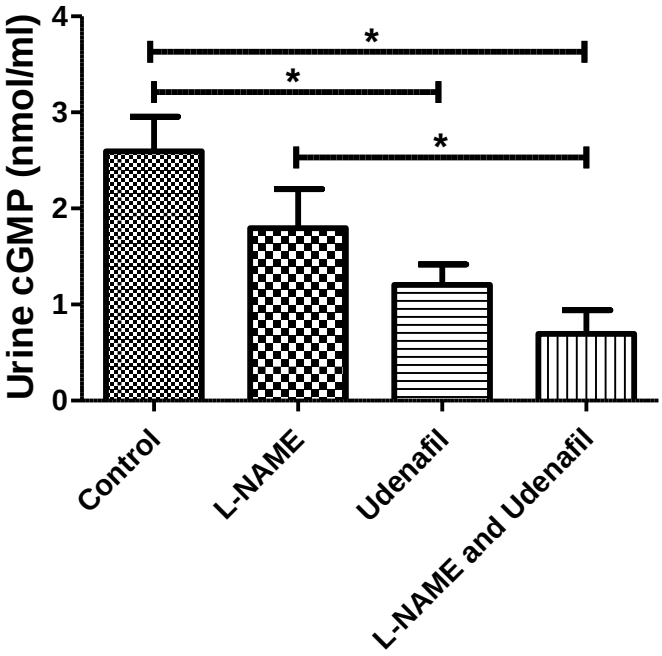


Figure 2. Comparison among the different groups of the effects on the nitric oxide system. Urine cGMP concentration levels were decreased in Udenafil compared to Control, L-NAME compared to L-NAME and Udenafil group. *$p < 0.05$

2. The comparison of renal function and degree of injury among the groups

In the Udenafil group, kidney function showed a slight increase as indicated by the decrease in serum creatinine. However, other groups did not show a statistically significant change (Fig 3). PCNA expression in terms of PCNA-positive tubular cells/unit area was 0.11±0.06, 0.29±0.17, 0.17±0.06, and 0.19±0.08 ml for the control, L-NAME, Udenafil, and L-NAME with Udenafil group, respectively ($p < 0.05$, control vs L-NAME, and L-NAME vs Udenafil and L-NAME with Udenafil) (Fig. 4). The urine NGAL levels were 279.8±126.8, 651.0±195.3, 473.7±114.9, and 326.5±279.4 ng/ml for the control, L-NAME, Udenafil, and L-NAME with Udenafil group, respectively ($p < 0.05$ control vs L-NAME and L-NAME with Udenafil) (Fig. 5). There were no functional changes in the kidney among the groups, but acute injury to tubular cells was well established in the L-NAME group, and this damage was ameliorated by udenafil treatment.

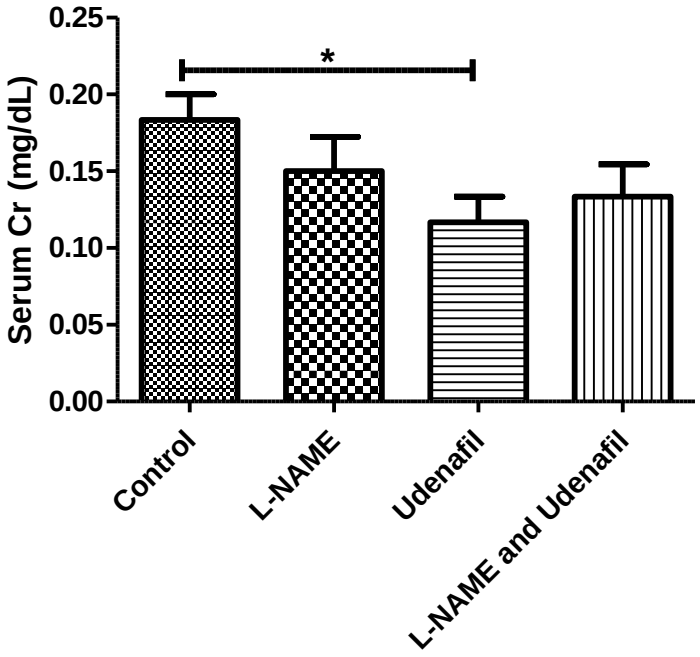
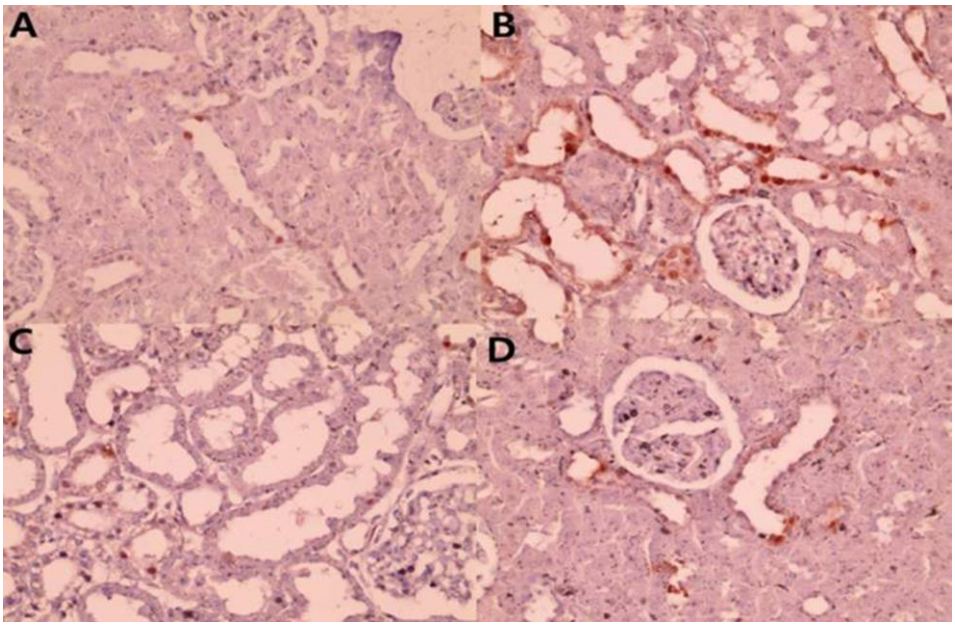


Figure 3. The comparison of serum creatinine levels among the groups. In the Udenafil group, kidney function was slightly increased as represented by decreased serum creatinine levels. However, other groups did not show statistically significant changes. *$p < 0.05$



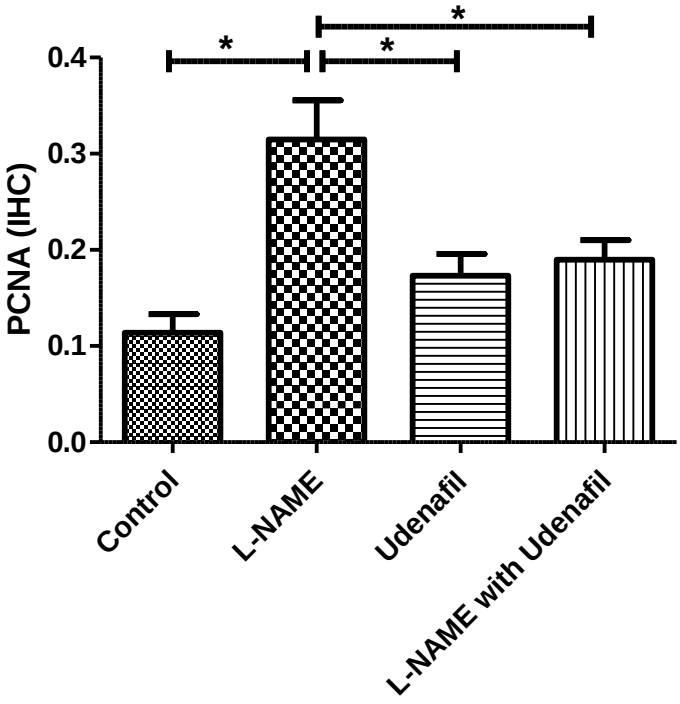


Figure 4. Comparison of proliferative cell nuclear antigen (PCNA) expression in immunohistochemical stain among the groups. (A) Control, (B) L-NAME, (C) Udenafil, (D) L-NAME with Udenafil. L-NAME group (B) showed the strongest response of PCNA positive nucleus in tubules. It was statistically significant. However, L-NAME with Udenafil group (D) was not different from control group (A). *$p < 0.05$

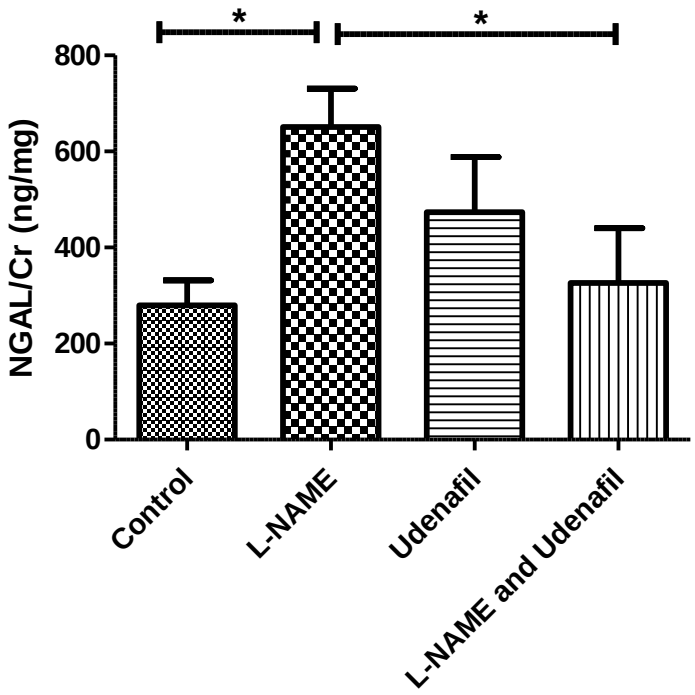


Figure 5. Comparison of the urine NGAL/Cr ratio among the groups. There were no functional changes of kidney among the groups. The urine NGAL concentration was highest in L-NAME group. However, L-NAME with Udenafil group was not different from control group. *$p < 0.05$

3. The changes of epithelial-mesenchymal transition markers after treatment

Alpha SMA showed increased expression in the L-NAME group compared to that the L-NAME with Udenafil group (control 0.45±0.02, L-NAME 0.95±0.05, Udenafil 0.50±0.02, and L-NAME with Udenafil 0.31±0.11). E-cadherin protein expression decreased in the L-NAME group compared to that in the other groups (control 0.911±0.01, L-NAME 0.36±0.09, Udenafil 0.99±0.01, and L-NAME with Udenafil 0.62±0.03) (Fig. 6). Considered together with the changes in PCNA expression, these results suggest that L-NAME treatment induced EMT in the kidney and that udenafil has a protective effect in the kidney.

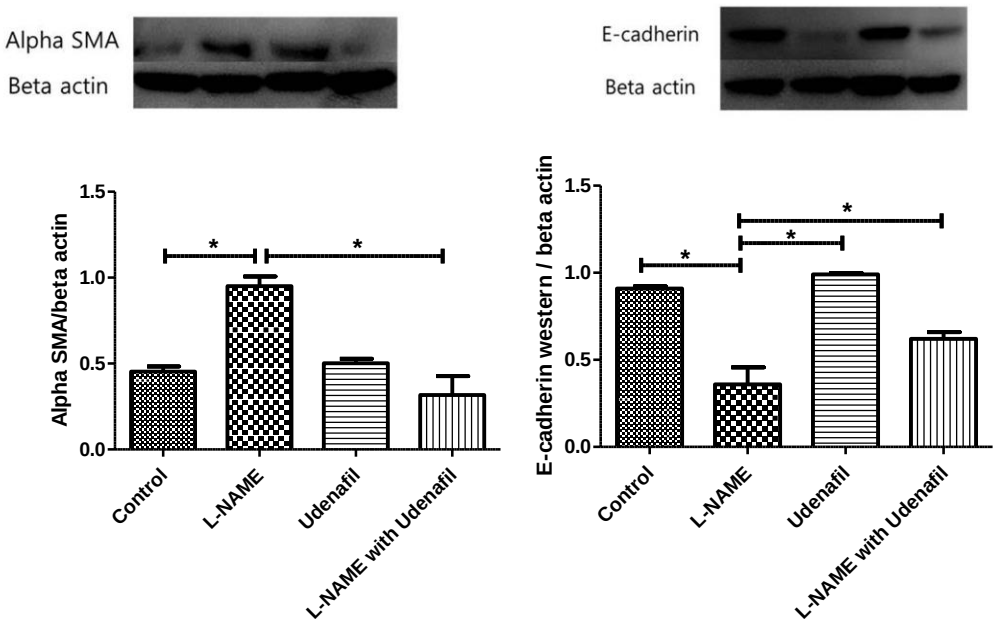
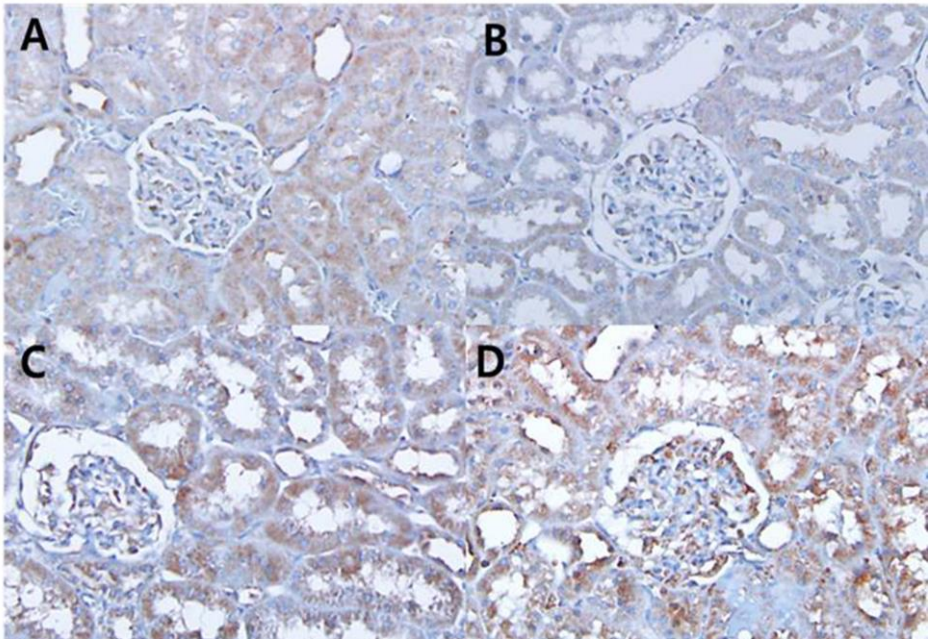


Figure 6. Comparison of kidney alpha SMA and E-cadherin expression among the groups as measured by western blot. Alpha SMA showed increased expression in the L-NAME group and decreased expression in the L-NAME with Udenafil group compared to control group. E-cadherin expression was lowest in the L-NAME group compared to control group. However, L-NAME with Udenafil group showed no difference from control group. *$p < 0.05$

4. The mechanism of attenuation of epithelial-mesenchymal transition after treatment

Klotho mRNA expression increased in the L-NAME with Udenafil group compared with that in the L-NAME group determined by RT-PCR (control 0.98±0.01, L-NAME 0.30±0.05, Udenafil 0.68±0.06, and L-NAME with Udenafil 0.54±0.13) (Fig. 7). Klotho protein density, as determined by immunohistochemical stain, showed the same trend among the groups. After treatment with udenafil, klotho mRNA and protein expression in tubular cells increased compared with that in the L-NAME group. Renal eNOS protein expression, measured by immunohistochemical staining, decreased in the udenafil group; however, eNOS protein expression in the L-NAME and L-NAME with Udenafil groups was not significantly different from that in the control (Fig. 8).



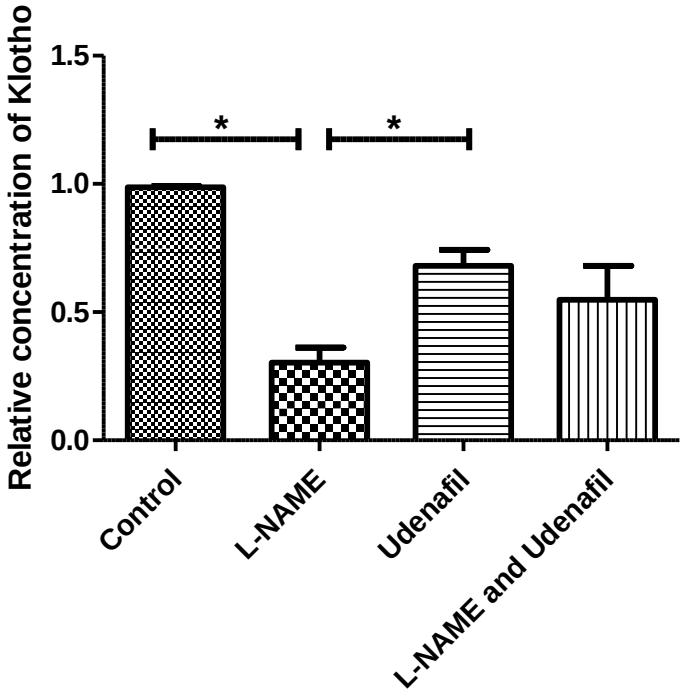
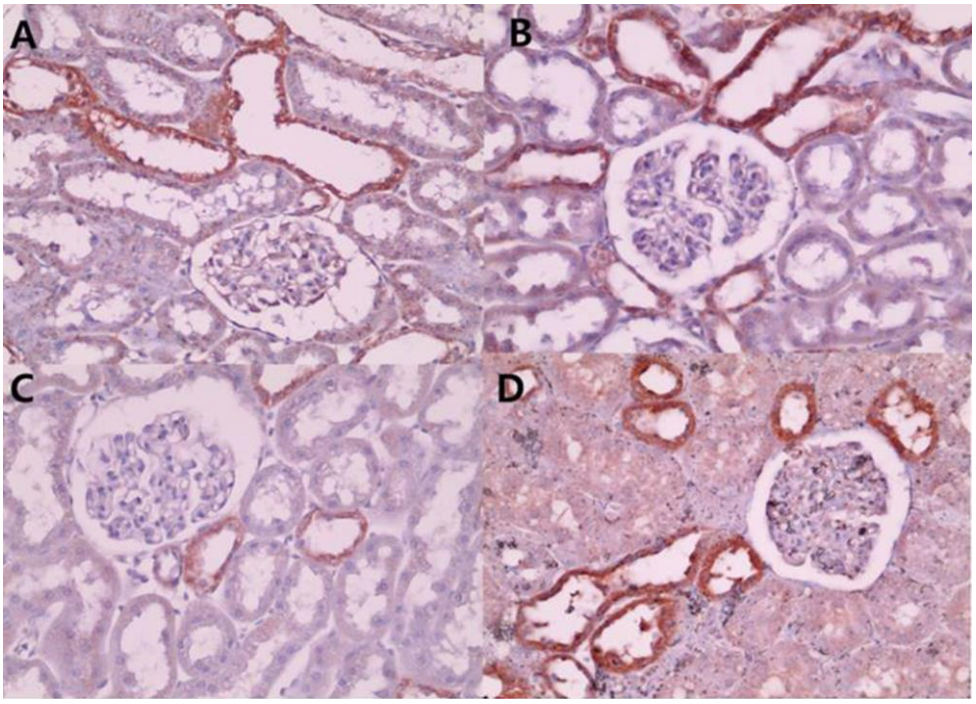


Figure 7. The comparison of protein density done by immunohistochemical stain and Klotho mRNA expression determined by RT-PCR in the kidney among the groups. (A) Control, (B) L-NAME, (C) Udenafil, (D) L-NAME with Udenafil. In the immunohistochemical stain, Klotho protein density showed same trend among the groups. After udenafil treatment, klotho mRNA and protein expression in tubular cells were increased compared to the L-NAME group. *$p < 0.05$



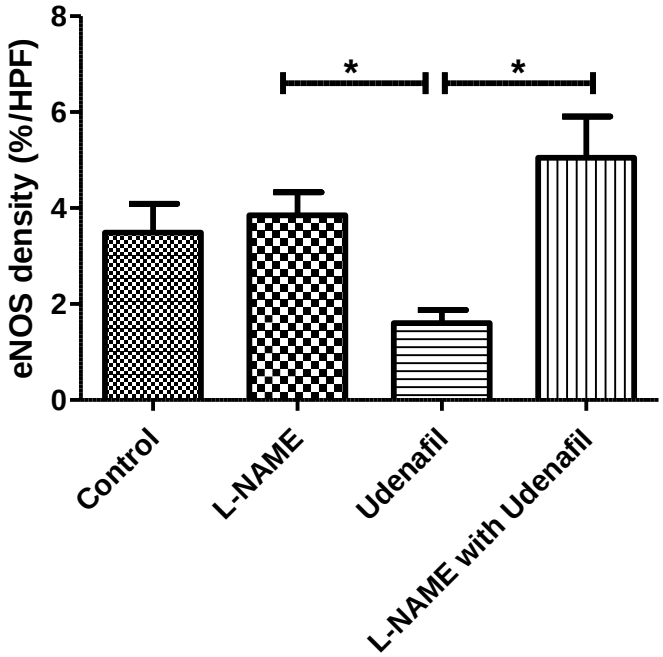


Figure 8. Comparison of eNOS protein expression in the kidney among the groups. (A) Control, (B) L-NAME, (C) Udenafil, (D) L-NAME with Udenafil. In the immunohistochemical stain, renal eNOS protein expression was decreased in the Udenafil group. However, eNOS protein expressions in the L-NAME and L-NAME with Udenafil group were not significantly different from the control group. $p < 0.05$

Ⅳ. DISCUSSION

Epithelial-mesenchymal transition (EMT) was induced by several factors in cancer cell and nitric oxide system had EMT in this study using L-NAME was not established well, however, Seccia etc. proved that endothelin-1 drives EMT in hypertensive nephroangiosclerosis (20). L-NAME also can make hypertension, podocyte injury and tubular damage in hypertensive rat model. There is no study measuring EMT markers in the kidney using L-NAME toxicity until now, we can confirm the change of E-cadherin, alpha SMA and PCNA in the tubular cell after treating L-NAME. Therefore, we can use L-NAME induced tubular injury model for EMT study.

PDE-5 inhibitors represent a class of drugs traditionally used to treat erectile dysfunction and pulmonary hypertension. Recent evidence suggests that PDE-5 inhibitors may have additional therapeutic effects such as cardioprotection and cerebrovascular protection (21). However, the direct effect and mechanism of PDE-5 inhibitors in renal fibrosis are not fully understood. Recently the long-acting PDE-5 inhibitor PF-00489791 was assessed in a multinational, multicenter, randomized, double-blind, placebo-controlled, parallel group trial of subjects with type 2 diabetes

mellitus and overt nephropathy receiving angiotensin converting enzyme inhibitor or angiotensin receptor blocker background therapy. Compared to placebo, 12-week treatment with PF-00489791 resulted in a significant reduction of 15.7% in the urinary albumin-to-creatinine ratio (22). Results of several animal studies suggested that PDE-5 inhibitors such as sildenafil, vardenafil, and tadalafil have a protective effect on renal ischemic-reperfusion injury (23-26). To demonstrate the protective effect of a PDE-5 inhibitor on an impaired NO system in the kidney, we postulated that the central molecule between EMT and cGMP would be eNOS. However, eNOS expression in the kidney tissue did not show significant difference among groups in our pilot study. Richter et al. suggested that klotho is the key molecule involved in the modulation of the NO system. In the presence of klotho, FGF23 induces NO release in human coronary artery endothelial cells, and its stimulating effects on ROS production are counterbalanced by increased ROS degradation. In states of klotho deficiency, FGF23-mediated NO synthesis is blunted and the rate of ROS formation exceeds that of ROS degradation (27). We focused on klotho expression in our animal model to prove the mechanism of PDE-5 inhibition under conditions of NO system deterioration.

Our animal model was designed to demonstrate the effect of cGMP

increase under impaired NO donation conditions in kidney tissue. One study showed the effects of peroral sildenafil administration in the macaque monkey (*Macaca fascicularis*) by performing chemical analysis of plasma and cerebrospinal fluid (CSF) using liquid chromatography coupled with tandem mass spectrometry. The results showed that drug levels in the CSF were high enough to inhibit PDE-5 activity, which was also demonstrated by the significant increases in CSF cGMP levels (28). In our animal model, urine cGMP levels decreased in the L-NAME with Udenafil group compared with that in the other groups; although the cGMP level in the kidney tissue was not quantified, the effect of PDE-5 inhibitor was enough to enable assessment of group differences.

CKD is a prevalent disease affecting 13% of adults in the United States, with 8% having an estimated glomerular filtration rate (eGFR) of <60 mL/min per 1.73 m^2 according to the National Health and Nutrition Examination Survey (1999–2004) (29). In recent years, considerable evidence has accumulated to suggest that CKD is a state of relative renal and systemic NO deficiency caused by a combination of decreased renal and vascular NO production and increased NO bioinactivation (30). Because of the important protective role played by NO in the health of both renal and cardiovascular systems, it is likely that a deficit in NO will

accelerate CKD progression and increase cardiovascular risk, making the NO pathway a promising therapeutic target (31).

The relationship between the NO system and klotho was not clearly elucidated in this study, but we showed that chronic NOS inhibition markedly reduced renal klotho protein expression. Moreover, a previous study showed that treatment with atorvastatin or pitavastatin completely prevented the reduction of klotho expression induced by NOS inhibition (32). Klotho protein exists in both a secreted and a membrane form. Its extracellular domain can be shed from the cell surface after cleavage by secretases and released into the circulation to act as an endocrine factor. Soluble klotho is a multifunctional protein present in biological fluids including blood, urine, and cerebrospinal fluid. It plays important roles in antiaging, energy metabolism, inhibition of Wnt signaling, anti-oxidation, modulation of ion transport, control of parathyroid hormone and active vitamin D production, and antagonism of the renin-angiotensin-aldosterone system (33). Soluble klotho and active vitamin D levels decrease and FGF23 levels increase at early CKD stages, whereas parathyroid hormone levels increase at more advanced CKD stages (34).

In the ischemia-reperfusion injury rodent model, klotho in the kidneys, urine, and blood decreased; klotho levels in all these organs were

restored upon recovery. Reduction in kidney and plasma klotho levels occurred earlier than reduction of NGAL, a known biomarker of kidney injury (35). Our study also showed that klotho mRNA and protein levels in the kidney were inversely correlated with NGAL expression, although the time sequence of this phenomenon was not clear. Klotho might have some beneficial effect on L-NAME-induced kidney injury.

Eryptosis, the suicidal death of erythrocytes, is characterized by erythrocyte shrinkage, blebbing, and phospholipid scrambling of the cell membrane. Eryptosis is enhanced in mouse models of sickle cell anemia and thalassemia, as well as in mice lacking functional annexin 7, cGMP-dependent protein kinase type I, AMP-activated protein kinase, Janus kinase 3, anion exchanger 1, adenomatous polyposis coli, or klotho (36, 37). Therefore, we postulated this mechanism was that deficiencies of cGMP and klotho can induce cell death and have interrelationship. Our study results showed that induction of cGMP independent of NO can restore klotho expression and reverse renal injury induced by L-NAME.

The origin and precise molecular and functional differences between mesenchymal cells and fibroblasts during EMT are not completely understood. Renal fibrosis is a pathological condition characterized by excessive accumulation of extracellular matrix and it is a common pathway

for the progression of different renal diseases. Transforming growth factor-β1 (TGF-β1) is implicated in the pathogenesis of fibrosis in both glomerular and interstitial compartments of the kidney. Klotho acts as an endogenous inhibitor of multiple growth factors including TGF-β1 (38, 39). In one study, renal fibrosis was induced by unilateral ureteral obstruction (UUO) in mice with reduced klotho expression (kl/+ mice) and they were then compared with wild-type mice. The UUO kidneys from kl/+ mice had significantly higher levels of fibrosis markers such as α-SMA, fibronectin, and TGF-β1 than those from wild-type mice (40). E-cadherin, a key component of cell–cell adhesion junctions, is essential for the formation of epithelia during embryonic development and for the maintenance of adult epithelial homeostasis; its loss is associated with increased tumor cell invasiveness (41). L-NAME-induced renal injury increased the levels of EMT markers such as PCNA and α-SMA, and decreased the levels of anti-EMT markers such as E-cadherin (42). Thus, EMT can be improved by a PDE-5 inhibitor treatment via klotho attenuation.

Kidney fibrosis induced by EMT was aggravated by reduced klotho levels, which can suppress TNF-α expression and attenuate NF-κB activation (43, 44). TGF-β1 mediated upregulation of PDE-4 activity promotes EMT in alveolar epithelial cells in lung tissue (45). One study

showed that the Klotho-induced reversal of EMT in SiHa cells is associated with downregulation of transcriptional factor Slug/Twist and resultant upregulation of E-cadherin (46). We also could demonstrate the same change of TWIST 1,2 mRNA (EMT signaling markers) in the kidney after treatment (data not shown). Until now, there is no study has demonstrated a direct connection between renal EMT and PDEs. We postulate that the core molecule of attenuating EMT after tubular injury involved might be klotho, because eNOS expression (NO system) was not changed after treatment (Table 1).

Table 1. Summary of the relationship between the nitric oxide (NO) system and epithelial-mesenchymal transition.

	Nitric oxide system	outcome	Klotho	eNOS	EMT		
					PCNA	E-cadherin	α SMA
L-NAME	↓	NO ↓ NO synthase ↓	↓	→	↑	↓	↑
L-NAME + cGMP phosphodiesterase inhibitor	↓	NO ↓ cGMP ↑ protein kinase G ↑	↔	↔	↔	↔	↔

NOS was inhibited by L-NAME in both of groups. However, in the L-NAME group, klotho was decreased and EMT markers were increased (PCNA and α-SMA). After PDE-5 inhibitor treatment, klotho was not changed and EMT markers were not changed, also.

V. CONCLUSION

In conclusion, we suggest that, in a poor NO environment, PDE-5 inhibitors can have protective effect on EMT via the klotho pathway independent of NO system.

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국 문 요 약

포스포디에스터 가수분해효소 5 억제제의 Klotho 발현 조절을 통한 신장 세뇨관의 상피-간엽 전환 완화 효과

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배경: 포스포디에스터 가수분해효소 5 억제제 (phosphodiesterase-5 inhibitor, PDE-5 inhibitor) 는 신체 내 여러 장기에서 cyclic guanosine monophosphate (cGMP) 의 분해를 통해 혈관을 확장시킨다. 그러나 정상적이지 않은 산화질소 (nitric oxide, NO) 시스템에서 PDE-5 inhibitor 의 작용은 아직 확실히 규명되지 않았다. 이전 연구에 따르면, 감소된 NO 생체이용률은 클로토 (klotho) 발현을 하향조절 시킨다고 했으나, klotho 와 NO 의 관계는 아직 명확하지 않다. 따라서 본

연구에서는 PDE-5 inhibitor 가 상피-간엽 전환 (epithelial – mesenchymal transition, EMT) 을 완화시킬 수 있는 지 여부 및 NO 와 klotho 의 관계를 알아보고자 한다.

방법: 200g 정도 무게의 10 주령 수컷 Sprague-Dawley rats (N=24) 를 사용하였다. 각 군은 대조군 (N=6), L-NAME (N=6), udenafil (N=6), L-NAME + udenafil (N=6) 으로 구성되었다. 그리고, 각 군에 따라 4 주간 L-NAME 1mg/mL 를 경구로 투여하거나, udenafil 5mg/kg 을 피하로 주사하였다. 연구 28 일째에 소변의 nitrate/nitritie, neutrophil gelatinase associated lipocalin (NGAL) 및 cGMP 를 측정하였다. 그리고 신장의 조직을 얻어 proliferative cell nuclear antigen (PCNA), α-smooth muscle cell antigen (α-SMA), E-cadherin 및 klotho 발현을 조사하였다.

결과: PDE-5 inhibitor 투여군에서는 cGMP 분해가 방해되면서 대조군에 비해 소변 내 cGMP 의 농도가 감소되었다. L-NAME 투여 군에서는 소변 내 NGAL 의 농도가 증가되었고 L-NAME, udenafil 동시 투여군에서는 증가되지 않고 대조군과 비슷하였다. EMT 표지자 인 PCNA 와 α-SMA 발현은 L-NAME 군에서 증가되었고 L-NAME, udenafil 동시 투여군에서는 감소되었다. 그러나, 항 EMT 표지자인 E-cadherin 경우에는 그 결과가 반대였다. Klotho 발현은 L-NAME 투여군에 비해 동시 투여군에서 증가되었다. 이와 같은 결과와 NO 시스템 과의 연관성을 알아보기 위해 endothelial nitric oxide synthase (eNOS) 발현을 측정하였는데, L-NAME 투여군과 동시 투여군을 비교해 볼 때 차이는 없었다.

결론: 본 연구는 L-NAME 투여군과 L-NAME · udenafil 동시 투여군의 NO 억제, 그리고 L-NAME 투여군에서 EMT 표지자의 변화와 klotho 감소가 L-NAME, udenafil 동시 투여 시 개선되는 결과를 근거로 하여, PDE-5 inhibitor 가 NO 시스템과 무관하게 klotho 를 통해 신장에서의 EMT 발생을 완화시킬 가능성을 제시하고 있다.

핵심 되는 말: 포스포디에스터 가수분해효소 5 억제제, 클로토, 상피-간엽 전환, 산화질소