Citrate Attenuates Tubulointerstitial Fibrosis in 5/6 Nephrectomized Rats by Decreasing Transforming Growth Factor- $_1$

Krissanapong Manotham MD*,
Somchai Eiam-Ong MD**, Somchit Eiam-Ong PhD***,
Pongsak Wannakrairot MD****, Kearkiat Praditpornsilpa MD**,
Saowaluck Chusil MD**, Kriang Tungsanga MD**

** Nephrology Unit, Medicine, Lerdsin General Hospital*
*** Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University Hospital*
**** Department of Physiology, Faculty of Medicine, Chulalongkorn University Hospital*
***** Department of Pathology, Faculty of Medicine, Chulalongkorn University Hospital*

Objective: *Tubulointerstitial fibrosis plays an essential role in progression to end stage renal disease (ESRD) in various chronic renal failure (CRF) models including the 5/6 nephrectomy (5/6). The present study examines the renoprotective effect of citrate in the renal ablative model that is quite similar to CRF in human.*
Material and Method: *Male Wistar rats underwent 5/6 and were fed with tap water ($5/6_{tap}$) or tap water containing 67 mEq/L citrate solution ($5/6_{cit}$). Sham-operated rats (S) were divided into S_{tap} and S_{cit} groups. Renal function, renal histopathology, renal α-Smooth muscle actin (SMA), and renal transforming growth factor (TGF) - $_1$ were determined immediately and at the 8^{th} week after operation.*
Results: *Following the surgery, the values of glomerular filtration rate (GFR) in the $5/6_{tap}$ and the $5/6_{cit}$ groups were 2.39 ± 0.25 and 2.35 ± 0.25 (mL/kg/min), respectively, both were significantly lower than sham groups ($p < 0.05$). At the eighth week, the $5/6_{tap}$ group had progressively decreased GFR and had higher fibrosis score, increased α-SMA positive cells, and renal tissue TGF- $_1$ when compared with the sham groups. The $5/6_{cit}$ group, when compared with the $5/6_{tap}$ group, had higher GFR (2.51 ± 0.22 vs 1.17 ± 0.33 mL/kg/min; $p < 0.05$), lower fibrosis score (1.83 ± 0.88 vs 3.0 ± 0.4, $p < 0.001$), lower -SMA activity (159 ± 2.9 vs 187 ± 12.3 cells per 1000 interstitial cells, $p < 0.05$), and lower renal TGF- $_1$ levels (1771.3 ± 239.5 vs 4716.9 ± 871.2 pg/mg protein, $p < 0.005$).*
Conclusion: *As such, in 5/6 nephrectomized rats, citrate therapy for eight weeks could decrease tubulointerstitial fibrosis mainly by reducing the heightened renal TGF- $_1$ levels and additionally by attenuating the increased myofibroblast activity.*

Keywords: *Citrate, 5/6 nephrectomy, Tubulointerstitisl fibrosis, TGF- $_1$*

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More than half a million people around the world are now on chronic dialysis and many folds of this number not receiving dialysis treatment have died due to end stage renal disease (ESRD)[1]. Renal fibrosis, especially in the tubulointerstitial area, plays an essential role in progression toward ESRD[2]. Halting the fibrosis is, thus, the highest hope to combat chronic renal failure (CRF). Expanding data indicate that interstitial fibrosis is a result of an imbalance between Extra cellular matrix (ECM) synthesis and degradation[2-4]. In both animals and humans with CRF, transforming growth factor - $_1$ (TGF- $_1$) appears to be the most po-

Correspondence to : Eiam-Ong S, Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University Hospital, Bangkok 10330, Thailand. Phone & Fax: 0-2252-6920, E-mail: EiamOng@netscape.net

tent fibrogenic growth factor that could induce ECM production and could reduce ECM metalloproteinase activity[5,6]. Myofibroblast, a specialized cell that is transdifferentiated from renal tubular cell[7], plays a major role in ECM synthesis. Myofibroblast transdifferentiation is upregulated by TGF- $_1$ but is mitigated by other cytokines including hepatocyte growth factor[8]. The major sources of renal TGF- $_1$ are macrophage and injured tubular as well as mesangial cells[9]. Several studies have demonstrated that interstitial fibrosis could be lessened by neutralizing the action of TGF-$_1$[10-13].

Citrate has been used for a long time as an alkali therapy in patients with renal tubular acidosis[14]. Apart from being a simple alkali donator, citrate is an important source of renal energy[15]. This could decrease ammonia production in proximal tubule[16,17] and could attenuate the redox state in the kidney[18]. Theoretically, citrate would have renoprotective effect in CRF. There are scarce data regarding the renoprotective effect of citrate in 5/6 neprectomized model, which is much similar to CRF in humans[19-21].

The present study was carried out to examine the effects of citrate on renal function, renal histopathology, myofibroblast transdifferentiation, and renal TGF- $_1$ levels in 5/6 neprectomized rats.

Material and Method

Animals

Male Wistar rats, between 4 and 6 weeks and 250 and 280 g, (The National Center of Science Use of Animals, Mahidol University, Salaya Nakorn Pratomp, Thailand) were used in the present study. The animals were given 5/6 nephrectomy (5/6) or sham operation. For 5/6, each rat was anesthetized with phenobarbital, 5 mg/100 g body weight, via intraperitoneal route. By dorsal approach, the left renal artery was identified and, 2 of 3 branches were ligated. In the right kidney, the right renal artery and the right ureter were ligated and removed. Four groups of animals were divided according to the types of drinking water.

1. 5/6 nephrectomized tap group ($5/6_{tap}$): tap water
2. 5/6 nephrectomized citrate group ($5/6_{cit}$): tap water containing citrate (67 mEq/L)
3. Sham tap group (S_{tap}): tap water
4. Sham citrate group (S_{cit}): tap water containing citrate (67 mEq/L)

From the pilot study, the animals taking such concentrations of citrate could maintain normal growth without any handicaps observed.

Experimental design

For the baseline data, after the surgery, all rats were placed in separate metabolic cages for a 24 hours urine collections. The tail blood samples were obtained to determine the glomerular filtration rate (GFR). All rats were, then, housed in the constant temperature of 24 C , 1-2 rats per housing, 12 hours light on-off controlled, and standard diet chow (protein 24%, fat 45%) ad libitum.

At the eighth week, all animals were separately placed in metabolic cages for the second 24 hour urine collections and GFR measurement. All the rats were then anesthetized with phenobarbital. By abdominal approach, the remnant kidney was dissected at the temperature of 4 C, washed in cold NSS until gross blood disappeared, and cut into two parts. For pathological and immunoperoxidase studies, tissue samples of the first part were fixed in 10% formalin and were further embedded in paraffin. For cytokine analysis, tissue samples in the other part were immediately dipped in liquid nitrogen and stored at -80 C till further analysis. Arterial blood samplings were drawn from the abdominal aorta to analyze blood pH and blood chemistry values.

Blood and urine samplings were analyzed for urea nitrogen, creatinine, and electrolytes by automated machine (Synchron Clinical System CX3, Beckman, USA). Arterial blood gas was measured by 238 pH/Blood Gas Analyzer, (Ciba Corning, England). The values of GFR were determined by the formulation: $GFR = (C_{urea} + C_{Cr})/2$, where C_{urea} = urea clearance and C_{Cr} = creatinine clearance.

Histopathological study

The kidney tissue samples for histological examination were fixed in 10% neutral-buffered formalin, then, embedded in paraffin block. Four micron sections were stained with standard hematoxylin and eosin, Periodic Acid Schiff (PAS), and Masson trichrome. The degree of fibrosis was evaluated and scored by the pathologist, in a blinded manner, according to Shih et al[22] on a scale of 0 to 4 (0 = normal; 0.5 = small focal areas of damage; 1 = involvement of less than 10% of the cortex; 2 = involvement of 10 to 25% of the cortex; 3 = involvement of 25 to 75% of the cortex; 4 = extensive damage involving more than 75% of cortex)

Immunohistochemistry study

Immunohistochemical study was carried out by microwave-stimulated technique to detect -smooth muscle actin (SMA), a myofibroblast marker[7,8]. In brief,

4 micron kidney tissues were deparaffinized and rehydrated with xylene, alcohol, and water. Endo-genous peroxidase was blocked with 0.5% H_2O_2. Antigen retrieval procedure was performed with citrate buffer and 2 steps microwave then stained with primary antibody to -SMA (col 4A1 mouse anti Human, Dako). The non specific staining was blocked with normal horse serum, then, incubated with bio-tinylated tag secondary antibody. The positive cells were characterized by cytoplasmic brown granular staining.

Two age- and size-matched rats were used as a positive control by performing Unilateral Ureteral Ligation (UUO) for a duration of 7 days. Their obstructed kidneys were stained for -SMA expression. Kidney tissue samples from two other young rats were also exploited as a negative control. The -SMA positive cells were strongly positive in positive control UUO rat. The mean of -SMA positive cells per 1,000 interstitial cells were 430 ± 20 cells. The positive -SMA cells mainly distributed in the interstitial area. On the contrary, the -SMA expression was rarely detected in negative control young rat and was present mainly in vascular wall. The distribution of -SMA positive cells found in the vessels was limited only in the media layer. The mean of -SMA positive cells per 1000 interstitial cells in young rats was only 45 ± 4 cells/ 1,000 interstitial cells.

Tissue TGF-$_1$

The -80 C storage tissues were thawed to 4 C then homogenized in 10 mM Tris-HCl (pH7.4), 2 M NaCl, 1 mM PMSF, 1 mM EDTA, and 0.01%Tween 80 buffer. The homogenated tissue specimens were centrifuged at 15,000 rpm 4 C for 30 minutes as previously described[6,9]. The supernatant part was acidified with 1 M acetic acid, urea, and was collected to measure TGF by Double antibodies ELISA (Quntikine kit™; R&D Systems).

Statistical analysis

All results were presented as mean ± SE. Data were analysed by analysis of variance (ANOVA) or unpaired T-tests where appropriate. The significant difference was attained when $p < 0.05$.

Results

Baseline data (week 0)

There were no significant differences in body weights among the experimental groups (Table 1). After the operation, both 5/6 groups had significantly decreased GFR compared with both S groups ($p < 0.05$, Fig. 1). No significant differences in GFR were noted between the S_{tap} and S_{cit} groups (5.19 ± 0.04 vs 4.96 ± 0.43 mL/kg/min) and between the $5/6_{tap}$ and $5/6_{cit}$ groups (2.39 ± 0.25 vs 2.35 ± 0.25 mL/kg/min).

The effect of citrate on 5/6 nephrectomized rats at the eighth week

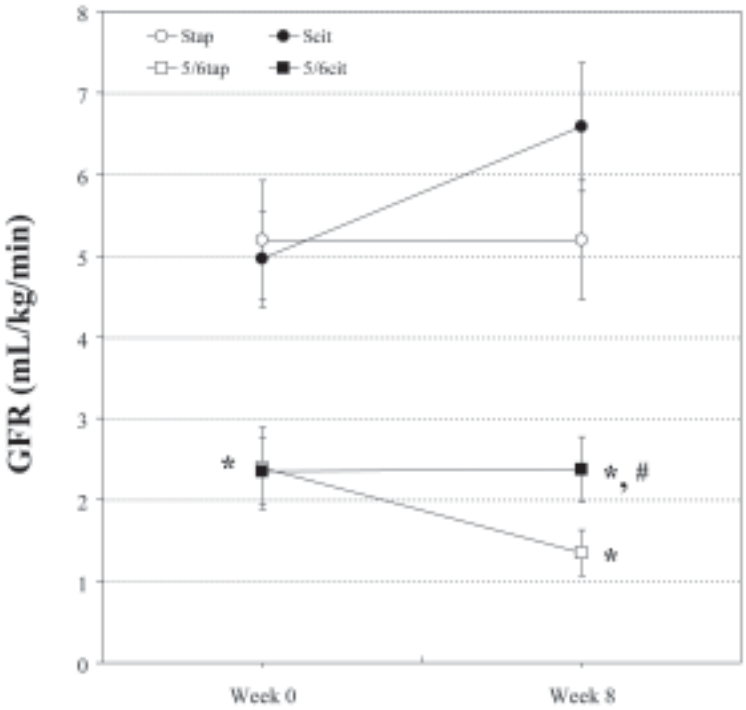
No significant differences were noted in body weight, blood pH, and electrolyte concentrations among the four experimental groups (Table 1).

As depicted in Figure 1, the mean GFR of the S_{cit} group was slightly higher, but not significantly, than the S_{tap} (6.58 ± 0.33 vs 5.19 ± 0.41 mL/kg/min). Animals in the $5/6_{tap}$ and $5/6_{cit}$ groups still had lower GFR than those in both S groups ($p < 0.05$). However, the $5/6_{tap}$ group showed a more progressive decrease in GFR than the $5/6_{cit}$ group (1.17 ± 0.33 vs 2.51 ± 0.22

Table 1. Body weight and blood parameters of animals in experimental groups

	S_{tap} (N = 4)	S_{cit} (N = 4)	$5/6_{tap}$ (N = 8)	$5/6_{cit}$ (N = 8)
Body weight (g)				
Week 0	282 ± 16	269 ± 13	261 ± 3	262 ± 9
Week 8	425 ± 3	419 ± 13	406 ± 22	397 ± 24
Blood parameters (week 8)				
pH	7.41 ± 0.05	7.43 ± 0.30	7.4 ± 0.04	7.45 ± 0.04
HCO_3^- (mEq/L)	23.7 ± 2.49	27.55 ± 0.75	26.5 ± 0.43	27.41 ± 1.5
Na^+ (mEq/L)	142 ± 0.9	141 ± 0.3	142 ± 0.9	141 ± 1.7
K^+ (mEq/L)	4.5 ± 0.1	4.5 ± 0.3	4.9 ± 0.5	4.7 ± 0.3
Cl^- (mEq/L)	106 ± 2	103 ± 3	105 ± 2	102 ± 2
Ca^{2+} (mEq/L)	9.7 ± 0.1	9.5 ± 0.2	9.7 ± 0.2	9.5 ± 0.1
PO_4^{3-} (mEq/L)	6.1 ± 0.3	6.7 ± 0.1	6.3 ± 0.3	6.5 ± 0.4

All values are given as mean ± SE. S = sham, cit = citrate, tap = tap water



*p < 0.05 vs Stap and Scit of the same period, #p < 0.05 vs 5/6tap of the same period

Fig. 1 The effect of citrate on glomerular filtration rate (GFR) in sham (n = 4) and 5/6 nephrectomized rats (n = 8)

mL/kg/min, $p < 0.05$).

Renal histopathologogical studies

Renal histopathologic studies in both S groups revealed minimal fibrosis. The mean fibrosis scores were 0.33 ± 0.15 and 0.25 ± 0.10 in the S_{tap} and S_{cit} groups, respectively (NS). Both 5/6 groups had higher pathological fibrosis scores than those of both S groups ($p < 0.001$, Fig. 2). Of interest, the $5/6_{cit}$ group had less fibrosis score than those of the $5/6_{tap}$ group (3.00 ± 0.37 vs 1.83 ± 0.4, $p < 0.05$).

Immunohistological study for smooth muscle actin

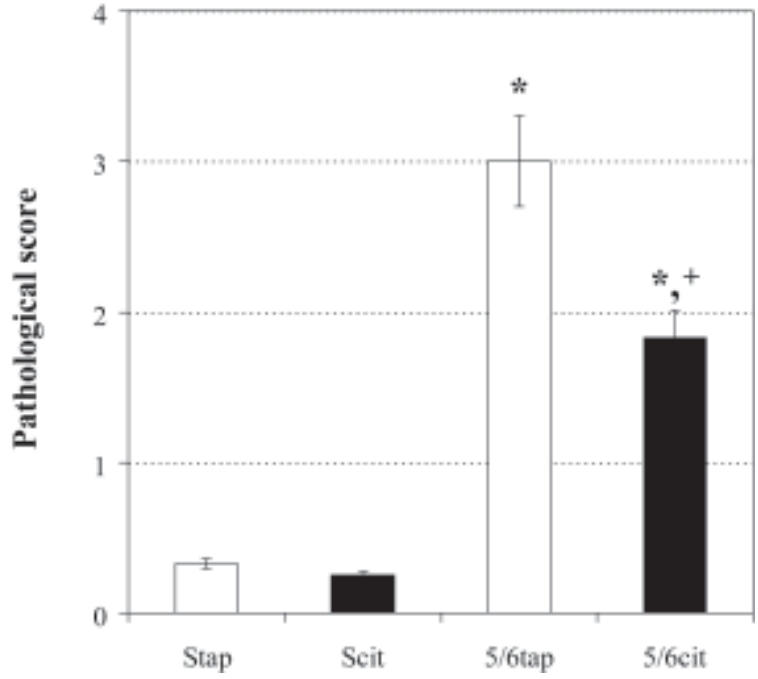
The S_{tap} and S_{cit} groups expressed minimal amounts of -SMA positive cells (65 ± 5 vs 58 ± 4 cells/1,000 interstitial cells, NS) (Fig. 3). Both values of -SMA positive cells were not different from the negative control group (45 ± 4 cells/1,000 interstitial cells). The cell distribution in the negative control was limited and more confined to the blood vessels (Fig. 4). A large amount of -SMA positive cells, elongated in shape like fibroblast, were detected in tubulointerstitial area in the $5/6_{tap}$ group ($p < 0.01$). In the $5/6_{cit}$, there were less -SMA positive cells (159 ± 3 vs 187 ± 12 cells/1,000 interstitial cells, $p < 0.05$). In the $5/6_{tap}$ group, the -SMA positive cells were not only distributed in the obviously fibrotic area, but were also noted in the adjacent area with inflammatory cell infiltration (Fig. 5). The distribution of these cells was more localized in the $5/6_{cit}$ group (Fig. 6).

Renal tissue TGF- $_1$ levels

The levels of renal tissue TGF- $_1$ in the S_{tap} and S_{cit} groups were $2,320.5 \pm 375.2$ and $1,668.2 \pm 56.7$ pg/mg protein, respectively (NS) (Fig. 7). The $5/6_{tap}$ group had much higher renal tissue TGF- $_1$ concentrations than the $5/6_{cit}$ group ($4,716.9 \pm 871.2$ vs $1,771.3 \pm 239.5$ pg/mg protein, $p < 0.005$) and those of both sham groups.

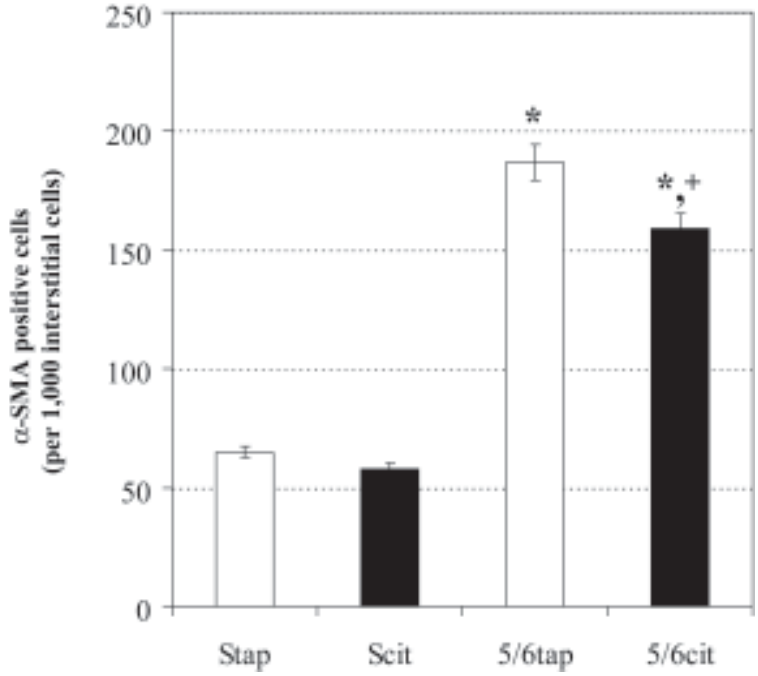
Discussion

The present study has shown that in 5/6 nephrectomized rats, the treatment with citrate for a duration of 8 weeks 1) could attenuate the progressive reduction in GFR, 2) could lessen tubulointerstitial fibrosis, 3) could reduce the increased -SMA positive cell



*p < 0.05 vs S_{tap} and S_{cit}, +p < 0.05 vs $5/6_{tap}$

Fig. 2 The effect of citrate on pathological score in sham (n = 4) and 5/6 nephrectomized rats (n = 8) at the eighth week



*p < 0.01 vs S_{tap} and S_{cit}, +p < 0.05 vs $5/6^{tap}$

Fig. 3 The effect of citrate on expression of α-smooth muscle actin (SMA) positive cells in sham (n = 4) and 5/6 nephrectomized rats (n = 8) at the eighth week

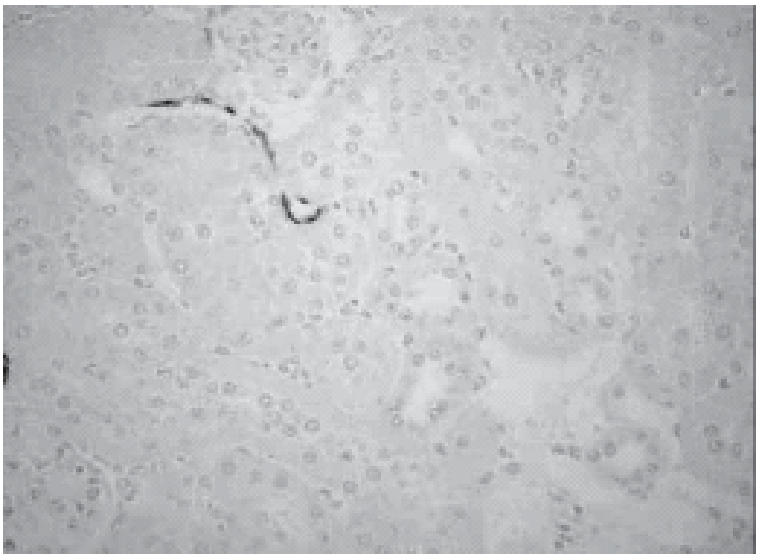
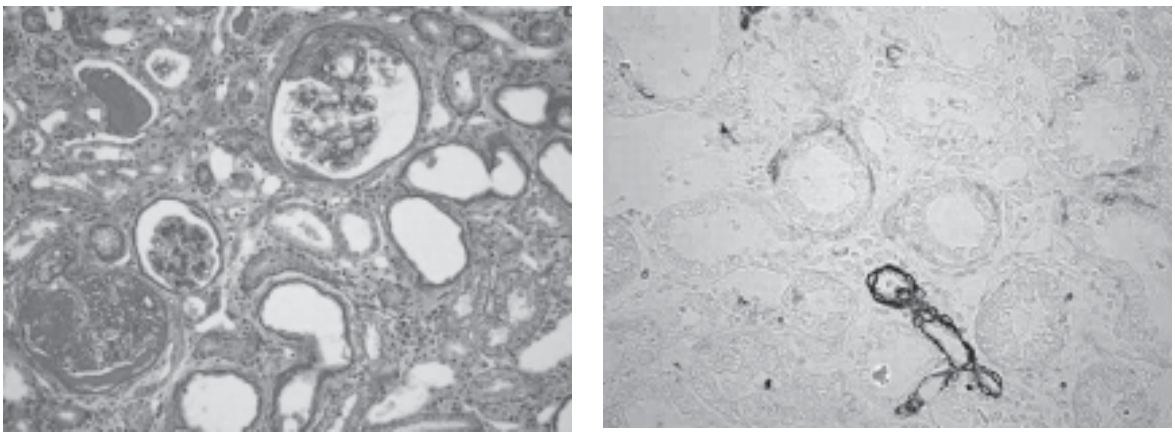


Fig. 4 Immunostaining for a-SMA in kidney of negative control. Only small vessels were positive for α-SMA. There was no remarkable fibrosis

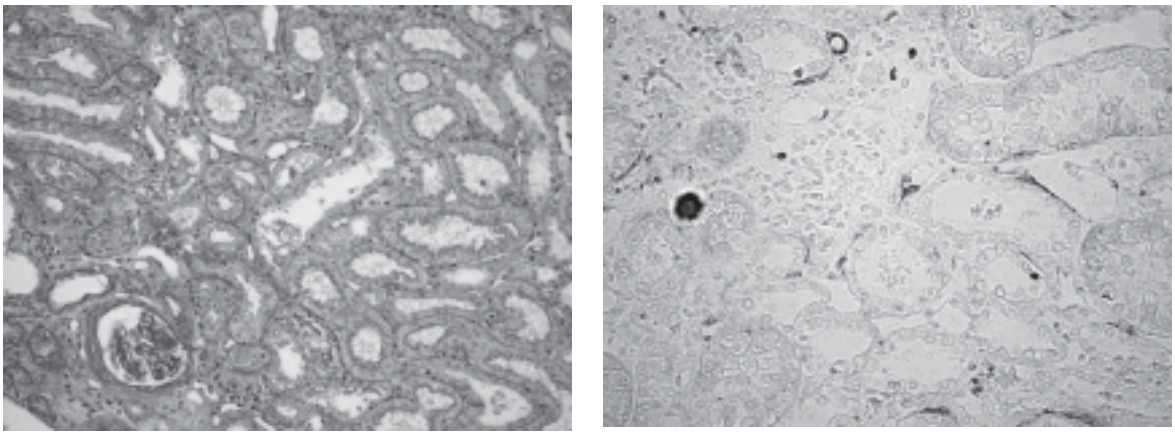


A **B**

Fig. 5 A) Kidney of 5/6 nephrectomized rat feeding with tap water (5/6 tap) showing wide irregular area of interstitial fibrosis associated with lymphocytic infiltration
B) Immunostaining for α-SMA in the same kidney in 5A α-SMA positive cells were prominent in interstitium

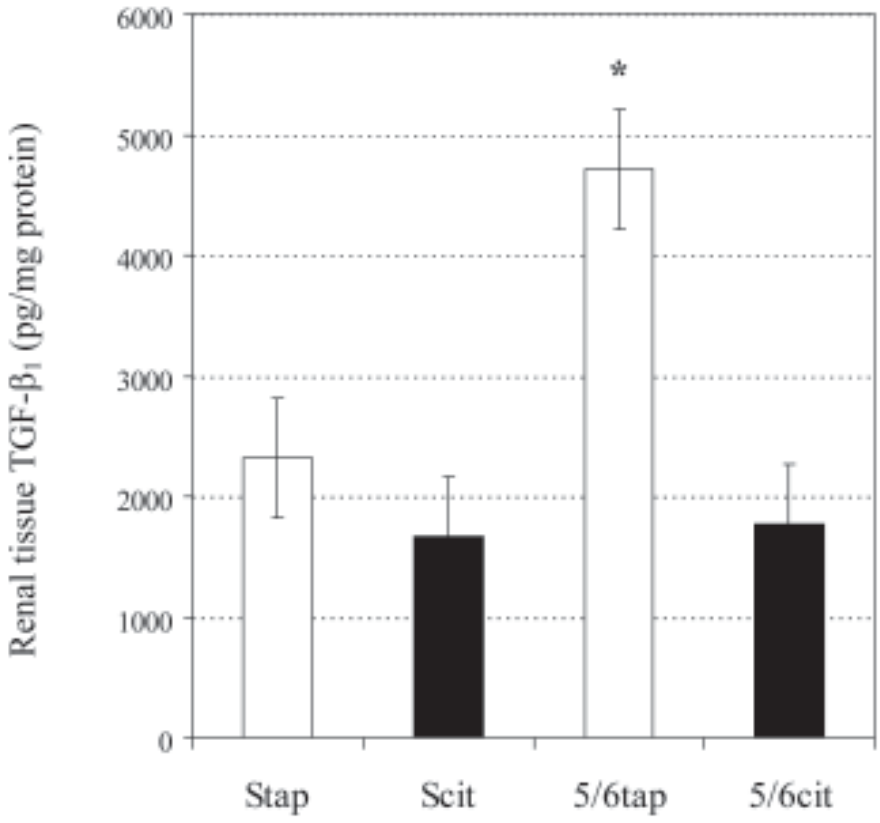
or myofibroblast numbers, and 4) could decrease the heightened renal tissue TGF- $_1$ levels. No alterations in systemic acid-base status and various serum electrolyte levels were observed.

Data regarding the renoprotective effect of citrate are sparsely available. Chronic ingestion of a solution of potassium citrate and citric acid improved renal function in the Han:SPRD rat. The Han:SPRD rat is a model of autosomal dominant Polycystic Kidney Disease (PKD)[16,17] but it had no renoprotective effect in the CD1-pcy/pcy mouse [23]. In a recent study, potassium citrate administration could ameliorate tubulointerstitial lesions in rats with uric acid nephropathy[24]. To the authors' knowledge, the present study is the first to demonstrate the renoprotective effect of citrate in 5/6 nephrectomized model, which is most frequently employed to study the events following extensive loss of functioning renal tissue and is considered to represent CRF in human. Of importance, the attenuation effect of citrate on progressively decreased GFR oc-



A B

Fig. 6 A) Kidney of 5/6 nephrectomized rat feeding with citrate in tap water (5/6 cit). Note that the interstitium was not widened
B) Immunostaining for α-SMA in the same kidney in 6A. Note that few α-SMA positive cells were focally seen in interstitium



*p < 0.005 vs S_{tap}, S_{cit}, and $5/6_{cit}$

Fig 7 The effect of citrate on renal tissue TGF-β_1 in sham (n = 4) and 5/6 nephrectomized rats (n = 8) at the eighth week

curs in association with reduction in tubulointerstitial fibrosis, -SMA positive cell numbers, and renal tissue TGF- $_1$ levels (Fig. 3, 6, 7).

Attenuation of the elevated renal tissue TGF- $_1$ levels and the increased -SMA positive cell numbers by citrate treatment, demonstrated in the present study, has also been noted in various renal injury models using other renoprotective therapies including angiotensin converting enzyme inhibitor[3], anti TGF- $_1$ antibody[12,13,25], and HMG CoA reductase inhibitor[26]. Of interest in the present work, the effect of citrate is more prominent on renal tissue TGF- $_1$ level than -SMA positive cell numbers (Fig. 3, 7). As such, the antifibrosis of citrate is likely mediated in the major part by decreased ECM degradation and in additional part via increased ECM synthesis.

Of note, the S_{cit} group had a tendency to have lower renal tissue TGF- $_1$ levels than the S_{tap} group although statistical significance was not attained (Fig. 7). Thus, citrate might interfere with the fibrogenesis pathway at the origin of cytokine production. Hence, no data have connected the citrate regulation in the kidney with TGF- $_1$production. Citrate might lessen renal tissue injury, one of the important proximate causes of increased TGF- $_1$production. In support of this contention, citrate is also an important source of renal energy[15]. Indeed, about 10% of renal energy comes from catabolism of citrate that occurs in proximal tubular cell. Besides, proximal tubule governs whole body acid regulation by generating ammonia[14]. Increased ammonia concentration, however, could lead to renal injury including tubuloin-terstitial fibrosis via complement activation as well as direct toxicity. Citrate has been reported to decrease proximal tubular ammonia production[16,17]. A recent study has demonstrated that citric acid cycle intermediates could correct mitochondrial dysfunction in proximal tubule during hypoxia/reoxygenation[18]. Such cellular protective effect of citrate in hypoxic conditions has also been shown in neuronal tissue[27].

Of interest in the present study were the values of GFR in the S_{cit} group that was slightly higher, although not significantly, than the S_{tap} group (Fig. 1). Since only minimal renal pathogy was noted in both sham groups, citrate might have direct positive effect on GFR. As such, the preservative effect of citrate on GFR in 5/6 nephrectomized rats could be mediated by the anti-fibrosis and, possibly, by the direct effect of citrate.

In the present work, the antifibrosis and renoprotective effects of citrate occurred without alterations in acid-base and electrolyte concentrations. Thus, the antifibrosis effect of citrate in 5/6 rats is not due to correction of acidosis observed in various renal injury models including citrate-treated Han:SPRD polycystic kidney rats[16,17]. Moreover, the role of acidosis in enhancing fibrosis has been challenged by the results in a recent study that demonstrated the antifibrosis effect of systemic acidosis in renal ablative model[28].

In conclusion, in 5/6 nephrectomized rats, citrate therapy could reduce the heightened renal TGF- $_1$ levels as well as the increased myofibroblast activity, leading to a decrease in tubulointerstitial fibrosis and, thus attenuation in decreased GFR. Citrate may also have a direct renoprotective effect on GFR. As such, citrate might be employed as the additional therapeutic agent in chronic renal failure patients.

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ซิเตรทลดภาวะทูบูโลอินเตอร์สทีเชียลไฟโบรซิสในหนูที่ถูกตัดไตห้าส่วนหก โดยผ่านกลไกการลดระดับทรานส์ฟอร์มมิ่งโกร๊ทแฟคเตอร์เบต้าวัน

กฤษณพงศ์ มโนธรรม, สมชาย เอี่ยมอ่อง, สมจิตร เอี่ยมอ่อง, พงษ์ศักดิ์ วรรณไกรโรจน์, เกื้อเกียรติ ประดิษฐ์พรศิลป์, เสาวลักษณ์ ชูศิลป์, เกรียง ตั้งสง่า

วัตถุประสงค์: *ทำการศึกษาผลในการป้องกันภาวะไตเสื่อมของซิเตรทในหนูที่ถูกตัดไตห้าส่วนหก ซึ่งเทียบเท่ากับภาวะไตวายเรื้อรังในคน*

วัสดุและวิธีการ: *แบ่งหนูวิสตาร์เพศผู้ที่ถูกตัดไตออกไปห้าส่วนหก เป็น 2 กลุ่มคือ กลุ่มที่ได้รับน้ำเปล่าและน้ำผสมกับซิเตรทที่มีความเข้มข้น 67 มิลลิอีควิวาเลนท์ต่อลิตร แบ่งหนูกลุ่มควบคุมออกเป็น 2 กลุ่มเช่นกัน ทำการตรวจหน้าที่ไต พยาธิสภาพของไต ระดับอัลฟ่าสมูธมัสเซิลแอคตินในไต ระดับทรานส์ฟอร์มมิ่งโกร๊ทแฟคเตอร์เบต้าวัน ที่เวลาเริ่มต้นและที่ 8 สัปดาห์*

ผลการศึกษา: *ที่เวลาเริ่มต้นการศึกษาพบว่า หนูที่ถูกตัดไตทั้งสองกลุ่มมีค่าการทำงานของไตลดลงอย่างมีนัยสำคัญ เมื่อเทียบกับกลุ่มควบคุมที่เวลา 8 สัปดาห์พบว่าหนูที่ถูกตัดไตและได้รับน้ำเปล่ามีค่าการทำงานของไตลดลงมากขึ้น มีการเพิ่มขึ้นของพยาธิสภาพทูบูโลอินเตอร์สทิเชียลไฟโบรซิส มีการเพิ่มขึ้นของระดับอัลฟาสมูธมัสเซิลแอคตินและทรานส์ฟอร์มมิ่งโกร๊ทแฟคเตอร์เบต้าวันในไตอย่างมีนัยสำคัญ ส่วนหนูที่ถูกตัดไตและได้รับซิเตรทมีความผิดปกติต่าง ๆ ดังกล่าวลดลงอย่างมีนัยสำคัญ*

สรุป: *การให้ซิเตรทเป็นเวลา 8 สัปดาห์แก่หนูที่ถูกตัดไตออกไปห้าส่วนหก สามารถลดความผิดปกติที่เกิดขึ้นกับหน้าที่ของไต พยาธิสภาพของไต ผลในการป้องกันภาวะไตเสื่อมของซิเตรทเกิดจากการลดระดับอัลฟ่าสมูธมัสเซิลแอคตินและระดับทรานส์ฟอร์มมิ่งโกร๊ทแฟคเตอร์เบต้าวันและฤทธิ์โดยตรงของซิเตรท*