วิตามินอีชนิดแอลฟาสามารถชะลอการเกิดพังผืดในไตที่เกิดจากการอุดตันของทางเดินปัสสาวะ: บทบาทจากการยับยั้งสัญญานของทีจีเอฟ-เบตาและสแมดที่เหนี่ยวนำการเปลี่ยนแปลงของเซลล์เยื่อบุผิวเป็นเซลล์เนื้อเยื่อยึดต่อ

นาย อดิศว์ ทัศณรงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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VITAMIN E (ALPHA TOCOPHEROL) AMELIORATES RENAL FIBROSIS IN URETERAL OBSTRUCTION: ROLE OF INHIBITING TGF-β /SMAD SIGNAL INDUCED EPITHELIAL-TO-MESENCHYMAL TRANSITION

Mr. Adis Tasanarong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
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Thesis Title	VITAMIN E (ALPHA TOCOPHEROL) AMELIORATES RENAL FIBROSIS IN URETERAL OBSTRUCTION: ROLE OF INHIBITING TGF-β /SMAD SIGNAL INDUCED EPITHELIAL-TO-MESENCHYMAL TRANSITION
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อดิศว์ ทัศณรงค์ : วิตามินอีชนิดแอลฟาสามารถชะลอการเกิดพังผืดในไตที่เกิดจากการอุดตันของทางเดินปัสสาวะ: บทบาทจากการยับยั้งสัญญานของทีจีเอฟ-เบตาและสแมดที่เหนี่ยวนำการเปลี่ยนแปลงของเซลล์เยื่อบุผิวเป็นเซลล์เนื้อเยื่อยึดต่อ. (VITAMIN E (ALPHA TOCOPHEROL) AMELIORATES RENAL FIBROSIS IN URETERAL OBSTRUCTION: ROLE OF INHIBITING TGF-β /SMAD SIGNAL INDUCED EPITHELIAL-TO-MESENCHYMAL TRANSITION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.สมชาย เอี่ยมอ่อง, 81 หน้า.

การเปลี่ยนแปลงของเซลล์เยื่อบุผิวเป็นเนื้อเยื่อยึดต่อเป็นกลไกที่สำคัญที่สุดที่เหนี่ยวนำให้เกิดความก้าวหน้าของการเกิดพังผืดในไต โดยได้รับการเหนี่ยวนำจากกลุ่มสัญญาณของ TGF-β และ Smad ร่วมกันในการแสดงบทบาทที่จำเป็นระหว่างที่เกิดขบวนการอักเสบในกลไกนี้ วิตามินอีชนิดแอลฟาได้รับการศึกษาว่ามีคุณสมบัติต้านสารอนุมูลอิสระและต้านการอักเสบ ดังนั้นจากคุณสมบัติต้านการอักเสบของวิตามินอีชนิดนี้ จึงได้ถูกนำมาทำการทดสอบในการศึกษานี้ เพื่อดูผลในการต่อต้าน (1) ความก้าวหน้าของการเกิดพังผืดในไต โดยใช้หนูที่ได้รับการเหนี่ยวนำให้เกิดการอุดตันของทางเดินปัสสาวะอย่างสมบูรณ์ของไตเพียงข้างเดียว (2) การเหนี่ยวนำจากกลุ่มสัญญาณของ TGF-β และ Smad ในการเหนี่ยวนำการเปลี่ยนแปลงของเซลล์เยื่อบุผิวเป็นเนื่อเยื่อยึดต่อ โดยหนูที่ได้รับการผ่าตัดเหนี่ยวนำให้เกิดการอุดตันของทางเดินปัสสาวะเพียงข้างเดียว หรือ การผ่าตัดหลอก จะได้รับวิตามินอีทางปากขนาด 250 มิลลิกรัมต่อกิโลกรัมเทียบกับยาหลอก โดยจะได้รับยาทุกวัน ตั้งแต่ 5 วันก่อนที่จะถูกเหนี่ยวนำให้เกิดการอุดตันของทางเดินปัสสาวะต่อจนกระทั่ง 14 วัน หลังจากการทำหัตถการ หนูเหล่านี้จะได้รับพิสูจน์ทราบในวันที่ 3 วันที่ 7 และวันที่ 14 หลังจากการทำหัตถการ โดยนำไตที่ได้ไปทำการศึกษาทางพยาธิวิทยาและการตรวจทางห้องปฏิบัติการด้วยการย้อม immunohistochemistry การวิเคราะห์โดย western blot และการตรวจด้วยวิธี real time RT-PCR เพื่อดูสัญญาณของ TGF-β, Smad และ เครื่องหมายของเนื้อเยื่อยึดต่อ ผลจากการย้อมชิ้นเนื้อไตด้วยวิธี H&E และ Masson trichrome พบว่าไตของหนูที่ได้รับเหนี่ยวนำให้เกิดการอุดตันของทางเดินปัสสาวะที่ได้รับยาหลอกเกิดความก้าวหน้าของการฝ่อเหี่ยวของเซลล์ท่อไตและพังผืดในไตเมื่อเทียบกับกลุ่มผ่าตัดหลอก อย่างไรก็ตามการรักษาด้วยวิตามินอีในหนูเหล่านี้ ทำให้การฝ่อเหี่ยวของเซลล์ท่อไตและพังผืดในไตดีขึ้นเมื่อเทียบกับกลุ่มที่ได้รับยาหลอก และจากการตรวจทางห้องปฏิบัติการเพื่อดูปริมาณของโปรตีนและยีนแสดงให้เห็นการเพิ่มขึ้นของ S100A4 เป็นเครื่องหมายของเซลล์เนื้อเยื่อยึดต่อ ซึ่งเป็นผลจากการเพิ่มขึ้นของ TGF-β1 และ Smad2/3 ร่วมกับพบการลดลงของ BMP-7 และ Smad 1/5/8 ในไตหนูที่ได้รับเหนี่ยวนำให้เกิดการอุดตันของทางเดินปัสสาวะ ในทางตรงกันข้ามหนูกลุ่มที่ได้รับการรักษาด้วยวิตามินอีสามารถยับยั้งการเพิ่มขึ้นของ S100A4 TGF-β1 และ Smad2/3 แต่ยังคงรักษาไว้ซึ่งการลดลงของ BMP-7 และ Smad 1/5/8 สรุปได้ว่าการรักษาด้วยวิตามินอีทำให้ความก้าวหน้าของการเกิดพังผืดในไตของหนูที่ได้รับการเหนี่ยวนำให้เกิดการอุดตันของทางเดินปัสสาวะดีขึ้น โดยการยับยั้ง TGF-β1 และ Smad2/3 แต่ยังคงรักษา BMP-7 และ Smad 1/5/8 ไว้ ในระหว่างที่เกิดการเปลี่ยนแปลงของเซลล์เยื่อบุผิวเป็นเนื้อเยื่อยึดต่อ เชื่อว่าผลของการป้องกันการเสื่อมของไตโดยวิตามินอีนี้ จะนำมาซึ่งการรักษาที่มีคุณค่าในมนุษย์ เพื่อยับยั้งความก้าวหน้าของการเกิดพังผืดในไต

สาขาวิชา ...ชีวเวชศาสตร์... ลายมือชื่อนิสิต.....อดิศว์ ทัศณรงค์.....

ปีการศึกษา .....2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก...สมชาย เอี่ยมอ่อง

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KEYWORDS : VITAMIN E / TGF-β / SMAD SIGNAL / EPITHELIAL-TO-MESENCHYMAL TRANSITION

ADIS TASANARONG: VITAMIN E (ALPHA TOCOPHEROL) AMELIORATES RENAL FIBROSIS IN URETERAL OBSTRUCTION: ROLE OF INHIBITING TGF-β /SMAD SIGNAL INDUCED EPITHELIAL-TO-MESENCHYMAL TRANSITION. ADVISOR: PROF. SOMCHAI EIAM-ONG, M.D., 81 pp.

Epithelial-to-mesenchymal transition (EMT) is the most important mechanism that induces the progression of renal fibrosis. Transforming growth factor-β (TGF-β) superfamily/Smad signal transduction plays the critical roles during inflammatory process in EMT. Vitamin E (alpha tocopherol) is demonstrated to be anti-oxidant and anti-inflammatory property. So, the anti-inflammatory effect of vitamin E was examined in this study to against (1) the progression of renal fibrosis in mice with complete unilateral ureteral obstruction (UUO) and (2) the TGF-β superfamily/Smad signal transduction induced EMT. UUO or sham operation was induced in ICR mice and vitamin E (250mg/kgBW) or vehicle was administrated orally everyday from 5 day before until day 14 post operation. Mice were sacrificed at day 3, 7 and 14 after operation. Histopathology, TGF-β superfamily/Smad signals and mesenchymal markers were evaluated by immunohistochemical staining, western blot analysis and real time RT-PCR. Compared with sham group, H&E and Masson trichrome staining showed the progression of tubular atrophy and interstitial fibrosis (TA/IF) in UUO with vehicle treatment group. However, vitamin E treatment significantly ameliorated the TA/IF when compared with vehicle treatment. From protein and mRNA analysis revealed the increased expression of S100A4 that consequently from increased TGF-β1 and Smad2/3 but decreased expression of BMP-7 and Smad1/5/8 in the obstructed kidneys. In contrast, vitamin E treatment significantly inhibited the expression of S100A4, TGF-β1 and Smad 2/3 but maintained the expression of BMP-7 and Smad 1/5/8. In conclusion, vitamin E treatment ameliorated the progression of renal fibrosis in obstructed kidneys by inhibited TGF-β1 and Smad2/3 but maintained BMP-7 and Smad1/5/8 during EMT. Thus, the renoprotective effect of vitamin E could have therapeutic value in humans to inhibit the progression of renal fibrosis.

Field of Study : Biomedical Sciences Student's Signature Adis Tasanarong

Academic Year : 2010 Advisor's Signature Somchai Eiam-Ong

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CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CKD	Chronic kidney disease
ESRD	End stage renal disease
TA	Tubular atrophy
IF	Interstitial fibrosis
EMT	Epithelial-to-mesenchymal transition
TGF-β	Transforming growth factor beta
TGF-β1	Transforming growth factor beta1
BMP-7	Bone morphogenetic protein-7
TEC	Tubular epithelial cell
AKI	Acute kidney injury
UUO	Unilateral ureteral obstruction
ECM	Extracellular matrix
GFR	Glomerular filtration rate
PTC	Peritubular capillary

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Chronic kidney disease (CKD) is becoming a major public health problem worldwide (1-3). The current burden of disease might due to a change of the pathogenesis of CKD. Given the pathogenic progression of kidney disease, patients with CKD are at high risk for progression to the end stage renal disease (ESRD) which is the condition requiring dialysis or kidney transplantation to maintain patient's survival (4-6). The enormous costs of treatment lead to a large burden for the health care systems, particularly in developing countries. The incidence of CKD is increasing, with a doubling in the number of patients treated for ESRD in universal over the past decade (7, 8). Current concept of CKD treatment is prevention by life style modification and medication. Especially, blood pressure control, and decrease proteinurea are the target of treatment (9, 10). However, there is no definite therapeutic option to slow progression of CKD.

Many studies demonstrated that the development of interstitial fibrosis and tubular atrophy (IF/TA) is the main pathology in progression of renal fibrosis (11, 12). One of the main effector cells that contribute to the development of progressive renal fibrosis in CKD is the tubulointerstitial fibroblast. Importantly, a large proportion of the interstitial fibroblasts are known to be originated from the tubular epithelial cells (TEC) through the process of epithelial-to-mesenchymal transition (EMT) (13, 14). During the process of EMT, TEC lose their epithelial phenotype but acquire the mesenchymal phenotype, as well as the tubular basement membranes are disrupted. Consequently, this process induces renal tubular destruction and accumulation of myofibroblasts. Moreover, evidence pointing to the transforming growth factor β (TGF-β) superfamily of proteins is primary regulators of fibrosis (15). Indeed, TGF-β1 might be the most important fibrosis promoting cytokine in the kidney, whereas bone morphogenetic protein-7 (BMP-7) is thought to counteract the profibrotic activity of TGF-β1 (16). Upon binding of ligand, the constitutively active type II receptor activates the type I receptor

by phosphorylation. This activated receptor then phosphorylates downstream signaling effectors, called Smad proteins. BMP-7 mediated signaling activates receptors Smad 1, 5 and 8, whereas activin and TGF-β1 activate Smad 2 and 3 (17). These activated Smads interact with the common Smad 4, and this complex translocates to the nucleus to regulate target gene transcription. Two inhibitory Smad proteins, Smad 6 and 7 negatively regulate BMP-7 and TGF-β1 mediated signaling, respectively. These all could be the consequence of inflammatory processes induced EMT.

In contrast to the major role of EMT in contributing to the disease progression, several studies have suggested that EMT of the TEC could be reversible. Recent therapeutic trials in many animal models of acute kidney injury (AKI) and chronic renal failure have demonstrated the efficacy of many substances in preventing the progression of renal fibrosis. Some studies could show the reversible process of renal fibrosis (16). However, many clinical studies in human have been shown that several medications could not established the promising results in prevention and/or reverse the TA/IF in CKD patients. Therefore, from the animal and clinical point of view, it is important to study which intervention or drug therapy could potentially reverse or inhibit the EMT during the progression of CKD.

Vitamin E is the name given to be a family of eight molecules. They consist of two groups, tocopherols and tocotrienols, which contain saturated or unsaturated side chains. Vitamin E, particularly in the form of alpha tocopherol, has been proposed for the prevention or treatment of numerous health problems which is primarily due to its antioxidant and anti-inflammatory properties (18-21). In term of antioxidant capacity of vitamin E, it has been showed the benefit to treatment AKI and slows the progression of chronic kidney injury in many animal kidney disease models (19, 22). However, anti-inflammatory capacity of vitamin E has been demonstrated only in some kind of cell culture and animal models or clinical trial in coronary artery diseases. So, the author would like to prove the benefit of this vitamin in chronic kidney injury.

The present study tried to demonstrate the efficacy of vitamin E treatment in the slow progression of renal fibrosis by using unilateral ureteral obstruction (UUO) animal model. The author hopes that the prevention or amelioration of chronic kidney injury by

vitamin E might be the promising therapeutic approaches to the clinical treatment in CKD patients in the future.

1.2 Research questions

-Could vitamin E have anti-inflammatory effect to ameliorate renal fibrosis during chronic kidney injury?

-Could vitamin E inhibit EMT by suppressing pro-fibrotic TGF-β1/Smad 2/3 but preserving anti-fibrotic BMP-7/Smad1/5/8 signals?

1.3 Objectives

-To evaluate the efficacy of vitamin E could attenuate pathologic severity of TA/IF in obstructed kidneys.

-To evaluate the benefit of vitamin E could suppress pro-fibrotic TGF-β1/Smad signaling induced EMT in obstructed kidneys.

-To evaluate the benefit of vitamin E could preserve anti-fibrotic BMP-7 /Smad1/5/8 signaling during EMT in obstructed kidneys.

1.4 Hypothesis

The hypothesis of present study is inhibiting pro-fibrotic TGF-β1/Smad 2/3 but preserving anti-fibrotic BMP-7/Smad1/5/8 by anti-inflammatory property of vitamin E could be inhibit EMT and ameliorate fibrosis during chronic kidney injury.

1.5 Keywords

Vitamin E (alpha tocopherol) TGF-β/Smad 2/3

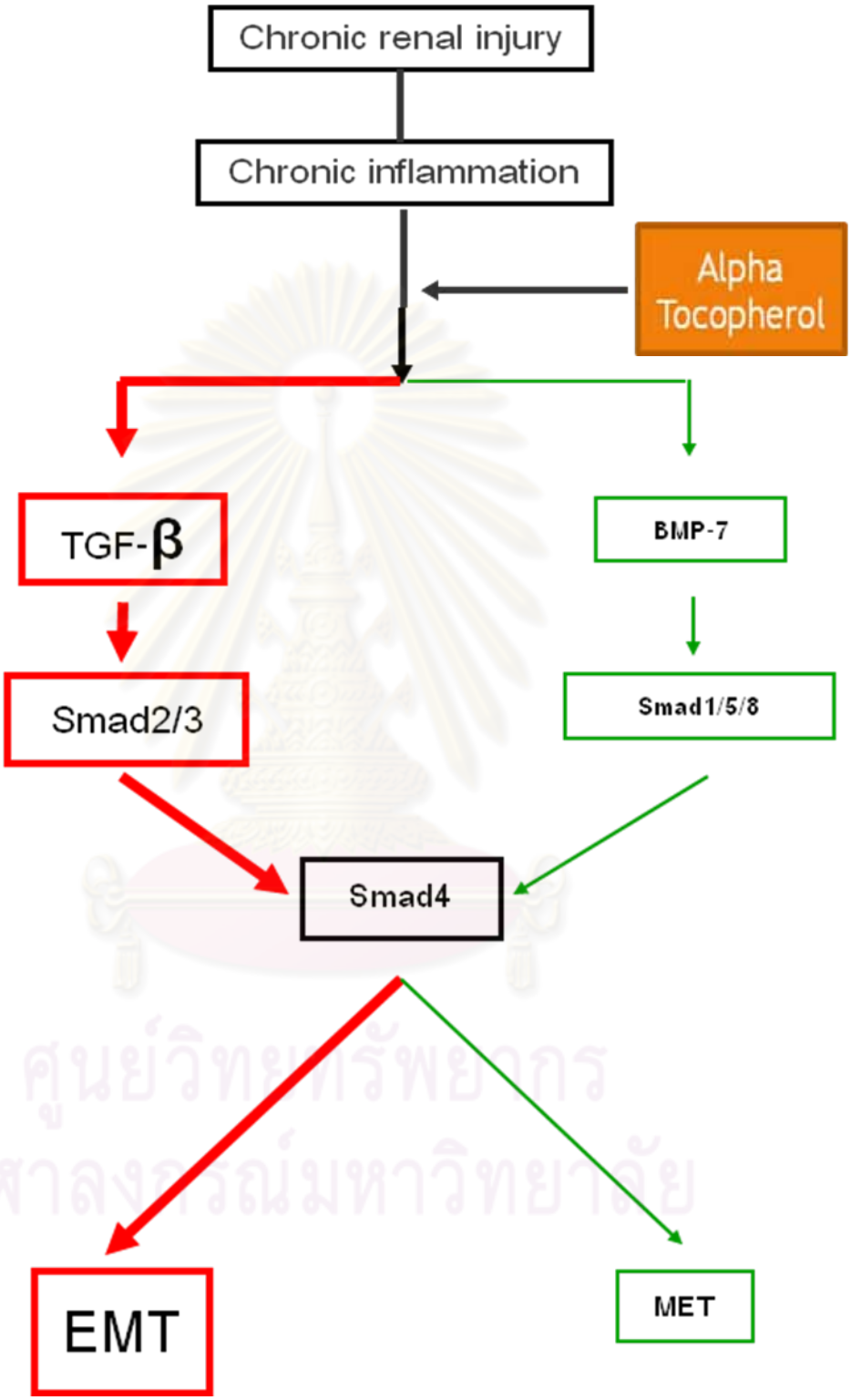
BMP-7/Smad 1/5/8 Epithelial-to-Mesenchymal Transition (EMT)

Fibroblast specific protein-1 (FSP-1), S100A4

1.6 Expected benefits and applications

The author tries to demonstrate the efficacy of the reno-protective effect by vitamin E to slow progression of CKD. The author hopes that the prevention or amelioration of chronic kidney injury by vitamin E might be the promising therapeutic approaches to the clinical treatment in CKD patients in the future.

1.7 Conceptual framework



CHAPTER II

LITERATURE REVIEW

CKD is one of the important public health problems around the world (1-3). Many reports demonstrated the rising incidence and prevalence of kidney disease, with poor outcomes and high therapeutic cost. Increasing evidence, in the past decades indicated that the adverse outcomes of CKD, such as kidney failure, cardiovascular disease, and premature death, can be prevented and delayed for renal replacement therapy (23, 24). Earlier stages of CKD can be detected through laboratory testing. Treatment of earlier stages of CKD is effective in slowing the progression toward to ESRD (25). Initiation of treatment for cardiovascular risk factors at earlier stages of CKD should be effective in reducing cardiovascular disease events both before and after the onset of kidney failure and could improve patient survival. Unfortunately, the problem in CKD patients is under-diagnosis leading to under-treatment, resulting in lost opportunities for prevention and disease advancement (26). One reason is the lack of agreement on a definition and classification of stages in the progression of CKD. A clinically applicable classification would be based on laboratory evaluation of the severity of kidney disease, association of level of kidney function with complications, stratification of risks for loss of kidney function and development of cardiovascular disease. Moreover, definite treatment to slow progression of CKD patients has not recovery yet. Although, in vivo studies demonstrated some medication could slow progression of CKD but they have not work properly in CKD patients (27-30).

2.1 Definition and classification of Chronic Kidney Disease (CKD)

CKD is a currently worldwide public health problem. Improving outcomes for people with CKD requires a coordinated global approach to prevention of adverse outcomes through defining the disease and its outcomes, estimating disease prevalence, identifying earlier stages of disease and antecedent risk factors, and detection and treatment for populations at increased risk for adverse outcomes. The operational definition and classification of stages of CKD provide an estimation of

disease prevalence by stage, to develop a broad overview of a “clinical action plan” for evaluation and management of each stage of CKD and to define individuals at increased risk for developing CKD also. Therefore, a simple definition and classification of kidney disease is necessary for international development and implementation of clinical practice guidelines. According to Kidney Disease: Improving Global Outcomes (KDIGO) has conducted a survey and sponsored a controversies conference to provide a clear understanding to both the nephrology and non-nephrology communities of the evidence base for the definition and classification recommended by Kidney Disease Quality Outcome Initiative (K/DOQI) (31, 32). Consequently, CKD is defined as kidney damage or glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for 3 months or more, irrespective of cause. Kidney damage in many kidney diseases can be ascertained by the presence of albuminuria, defined as albumin-to-creatinine ratio >30 mg/g in two of three spot urine specimens. GFR can be estimated from calibrated serum creatinine and estimating equations, such as the Modification of Diet in Renal Disease (MDRD) Study equation or the Cockcroft-Gault formula.

Table 2.1 Definition and classification of CKD (31, 32)

Chronic kidney disease is present if either of the following criteria is present for three months or more : 1. Structural or functional abnormalities of the kidney (with or without decreased GFR), as manifested by any of the following: - Pathological abnormalities - Markers of kidney damage *Proteinuria (albumin-to-creatinine ratio > 30 mg/g) *abnormalities of urine sediment *abnormal imaging studies *tubular syndromes - Kidney transplant recipient 2. GFR < 60 ml/min/1.73 m^2, with or without kidney damage.

Kidney disease severity is classified into five stages according to the level of GFR (31, 32). This global consensus for the adoption of a simple definition and classification of CKD could be have the advantage to identify a collaborative research agenda and plan that would improve the evidence base and facilitate implementation of the prevention and treatment in patients. Furthermore, uniform classifications of CKD by cause and by risks for kidney disease progression and CVD, kidney disease treatment by dialysis and transplantation should be developed soon.

Table 2.2 Stages of Chronic Kidney Disease (K/DOQI) (31, 32)

Stage	Description	GFR ($ml/min/1.73m^2$)
1	Kindney damage with normal or ↑GFR	≥ 90
2	Kindney damage with mild ↓GFR	60 - 89
3	Moderate ↓GFR	30 - 59
4	Severe ↓GFR	15 - 29
5	Kidney failure	< 15 (or dialysis)

2.2 Incidence and prevalence of CKD

CKD is now recognized as a common condition that elevates the risk of cardiovascular disease as well as ESRD and other complications requiring dialysis or transplantation. The current burden of disease might due to a change of the underlying pathogenicity of CKD. Current evidence suggests that hypertension and diabetes are the two major causes of kidney disease generally (33, 34). By K/DOQI definition and classification, the prevalence of CDK in all staging is increase in any time course in United States (35, 36). The National Health and Nutrition Examination Surveys (NHANES) have provided a rigorous basis for estimating CKD prevalence. Initial

prevalence estimation for CKD stages in NHANES 1988-1994 in adults have provided a benchmark for kidney disease studies, prevention efforts, and health care planning (37). Later studies have estimated NHANES 1988-1994 compared with NHANES 1999-2000 data found that an increased prevalence of albuminuria was not correlated with increase in the overall prevalence of CKD. The precision of these trend estimates was constrained by the relatively small sample size of the 1999-2000 survey and limited data to establish consistent calibration of the creatinine assays over time (38). Therefore, recent study by NHANES surveys from 1988 to 2004 showed that the prevalence of CKD in the United States in 1999-2004 is higher than it was in 1988-1994. This increase is partly explained by the increasing prevalence of diabetes and hypertension which raises concerns about future increased incidence of kidney failure and other complications of CKD (35). Moreover, data from USRDS 2000 Annual Data Report demonstrated that the incidence and prevalence of ESRD patients in the United States is increase and they can predict the number of ESRD patients in the future.

Table 2.3 Prevalence of Chronic Kidney Disease (CKD) stages in US adults (35)

CKD Stage[a]	Prevalence, % (95% CI)		Prevalence Ratio for NHANES 1999-2004 to 1988-1994 (95% CI)	Estimated No. of US Adults in 2000, No. in Millions (95% CI)
	NHANES 1988-1994	NHANES 1999-2004		
1	1.71 (1.28-2.19)	1.78 (1.35-2.25)	1.05 (0.85-1.30)	3.6 (2.7-4.5)
2	2.70 (2.17-3.24)	3.24 (2.61-3.88)	1.21 (1.03-1.41)	6.5 (5.2-7.8)
3	5.42 (4.89-5.95)	7.69 (7.02-8.36)	1.42 (1.25-1.62)	15.5 (14.1-16.8)
4	0.21 (0.15-0.27)	0.35 (0.25-0.45)	1.70 (1.11-2.51)	0.7 (0.5-0.9)
5	NA	NA	NA	NA
Total	10.03 (9.16-10.91)	13.07 (12.04-14.10)	1.30 (1.19-1.43)	26.3 (24.2-28.3)

Abbreviations: CI, confidence interval; NA, data not included because patients with CKD stage 5 were excluded; NHANES, National Health and Nutrition Examination Surveys.
[a] Defined based on standard criteria[1]: stage 1, persistent albuminuria with glomerular filtration rate (GFR) higher than 90 mL·min⁻¹·1.73 m⁻²; stage 2, persistent albuminuria with GFR of 60 to 89 mL·min⁻¹·1.73 m⁻²; stage 3, GFR of 30 to 59 mL·min⁻¹·1.73 m⁻²; stage 4, GFR of 15 to 29 mL·min⁻¹·1.73 m⁻². The age-adjusted prevalence rates for CKD stages 1, 2, 3, and 4 in 1988-1994 adjusted to the 1999-2000 age distribution in Table 1 are 1.7%, 2.8%, 5.6%, and 0.2%, respectively, for a total of 10.3%.

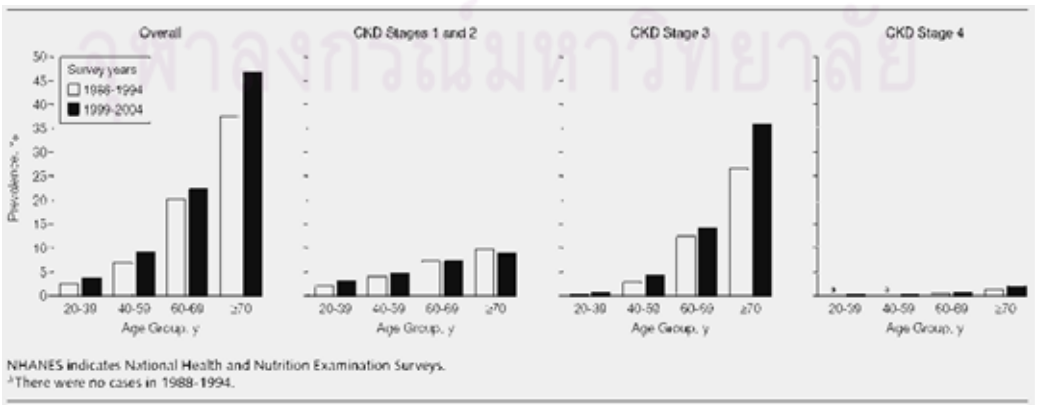


Figure 2.1 Prevalence of Chronic Kidney Disease (CKD) stages by age group (35)

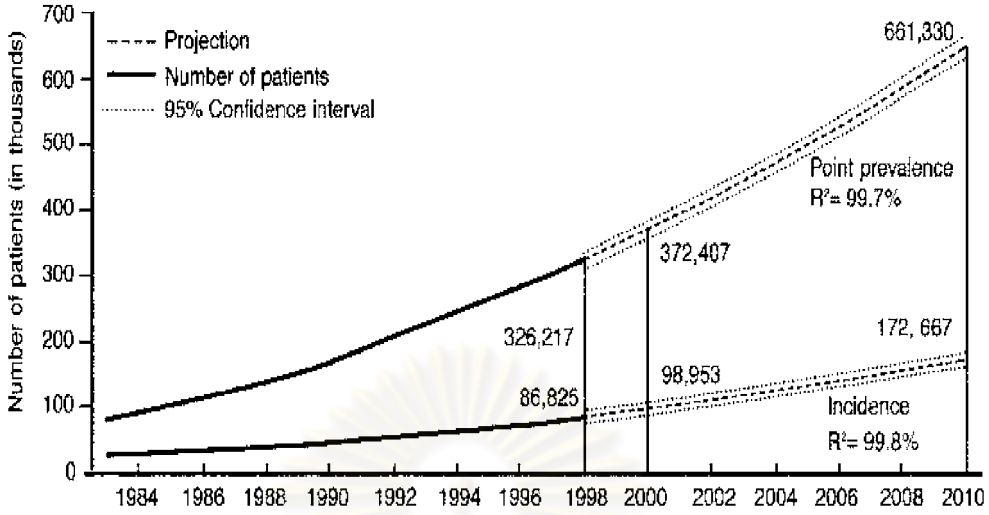


Figure 2.2 Incidence and prevalence of ESRD in the United States. (Data from USRDS 2000 Annual Data Report)

In addition, incidence and prevalence of CKD in Thailand have been assessment. Compared to the CKD prevalence reported from the United States, reports from Thailand showed that CKD prevalence fluctuated from a much lower to a high rate of occurrence ranged from 44.6% to 13.8% (39, 40). The population-based Thai Screening and Early Evaluation of Kidney Disease (SEEK) study was conducted with cross-sectional stratified-cluster sampling study. The 3,459 subjects were included in this study, and 626 subjects were identified as CKD follow K/DOQI classification. The evidence of overall CKD prevalence was 17.5%. The CKD prevalence of stages I, II, III and IV were 3.3%, 5.6%, 7.5% and 1.1%, respectively. CKD prevalence in the Thai population is much higher than previous study and early stages of CKD seem to be as common as later stages (41).

Table 2.4 Prevalence of CKD in the Thailand (39)

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Total
	GFR > 90 ± Proteinuria	GFR 60-89 ± Proteinuria	GFR 30-59	GFR 15-29	GFR < 15	
CCr* (C-G)	150 (1.0%)	94 (0.6%)	1135 (7.3%)	18 (0.1%)	8 (0.05%)	1405 (9.1%)
GFR** (MDRD)	128 (0.8%)	112 (0.7%)	460 (2.9%)	14 (0.1%)	9 (0.06%)	723 (4.6%)

* Creatinine clearance calculated by the Cockcroft-Gault formula
** Glomerular filtration rate calculated by the Modified Diet in Renal Disease (MDRD) Study equation

Table 2.5 Estimation of CKD prevalence according to gender (41)

		CKD staging									
		I		II		III		IV		Overall	
Gender	n	No.[a]	Prevalence[b] (%)	No.	Prevalence (%)	No.	Prevalence (%)	No.	Prevalence (%)	No.	Prevalence (%)
Male	1569	51	2.6 (1.4, 3.8)	100	5.8 (4.0, 7.6)	104	6.9 (4.9, 8.9)	15	0.9 (0.5, 1.3)	270	16.3 (12.5, 20.0)
Female	1890	83	3.8 (2.8, 4.9)	107	5.4 (3.5, 7.4)	144	8.0 (6.0, 9.9)	22	1.3 (0.6, 2.0)	356	18.5 (14.8, 22.3)
Overall	3459	134	3.3 (2.5, 4.1)	207	5.6 (4.2, 7.0)	248	7.5 (6.2, 8.8)	37	1.1 (0.7, 01.5)	626	17.5 (14.6, 20.4)
			8.9 (6.8, 11.0)				8.6 (7.0, 10.3)				

Given the pathogenic progression of kidney disease, patients with CKD are at high risk for progression to the end stage renal disease (ESRD). This condition requires dialysis or kidney transplantation to maintain patients' long-term survival. The enormous costs of treatment lead to a large burden for the health care systems, particularly in developing countries.

2.3 Pathology and progression of CKD

Despite from definite data, the most important problem in CKD patient is progressive loss of kidney function. Not only progression of CKD staging, resulting in death, dialysis, or a kidney transplant, but also a great propensity for several forms of cardiovascular disease are presented in CKD patients. The vascular calcification so common in kidney disease among those with reduced kidney function (42), develop excessive incidence of coronary artery disease in CKD. The role of reduced kidney function is also recognized as a risk factor for stroke. In addition, the important risk factors for CKD progression are proteinuria, diabetes, and high blood pressure (43-45), which are the role of other traditional and nontraditional factors.

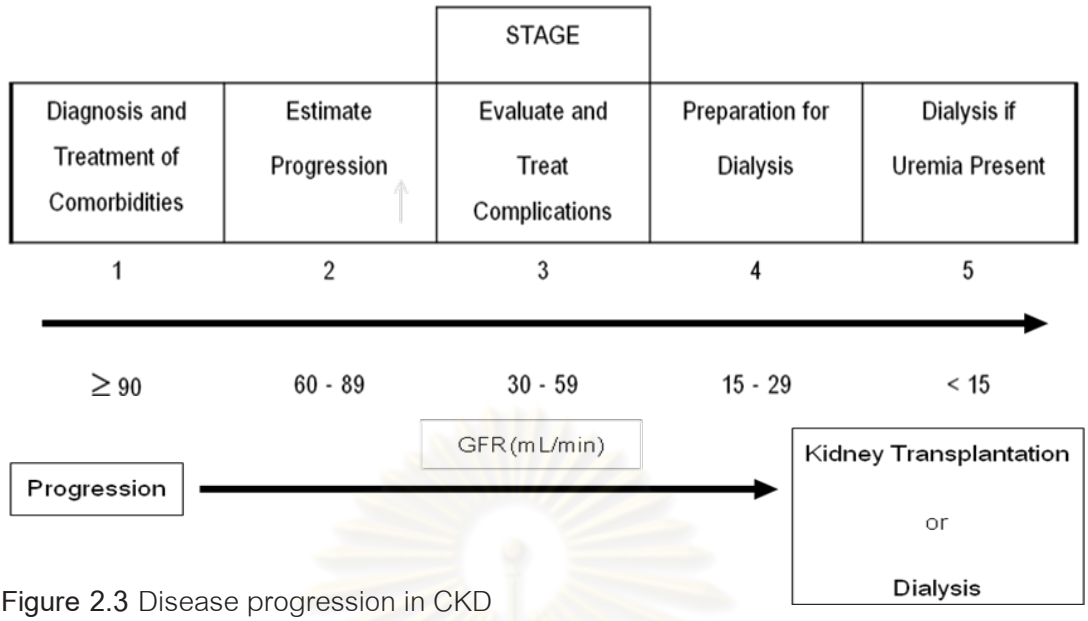


Figure 2.3 Disease progression in CKD

Therefore, almost risk factors as mentions could cause chronic inflammatory response in CKD patients, developing kidney disease progression. In the meantime, a series of basic science investigations continue to characterize the mechanisms of progression of kidney disease are the complex interactions among the glomerulus, TECs, interstitium and endothelial cell in vasculature of the kidney (46). There is compelling evidence that interstitial inflammation plays a central role in the loss of renal function in CKD (47). The combined effects of interstitial inflammation (12), oxidative stress (48) and local angiotensin II activity have implicated in the disruption of glomerulus and tubule continuity, the development of pathogenic hypoxia (49), the generation of myofibroblasts and fibrosis (50), and the impairment of the protective autoregulation of glomerular blood flow, leading to glomerulosclerosis. The association between proteinuria and progression of CKD is firmly established (51). For example, proximal tubular cells exposed to high concentration of proteins release pro-inflammatory and pro-fibrotic factors including nuclear factor Kappa-B (52) and the signal transducer and activator of transcription results in the upregulation of a variety of cytokines and chemokines. These activations become over-expression of adhesion molecules and interstitial infiltration of inflammatory cells into the kidney. As a result, fibrosis is promoted by release of TGF-β which induces myofibroblast formation and collagen deposition. Finally, the participation of vitamin D3 deficiency can promote the

development of tubulointerstitial fibrosis. The molecule 1,25-$(OH)_2D_3$ modulates peritubular capillary (PTC) proliferation, suppresses fibroblast activation and matrix production, reduces EMT (53) and downregulates the genes of the renin-angiotensin system, which are critical steps in the development of a scarred kidney.

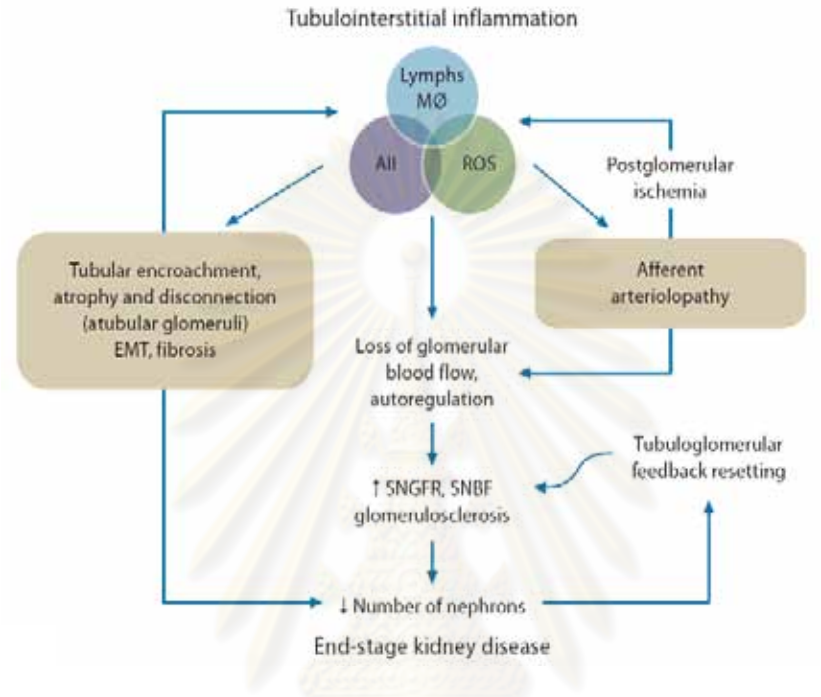


Figure 2.4 Mechanism of tubulointerstitial inflammation lead to disease progression in CKD (47)

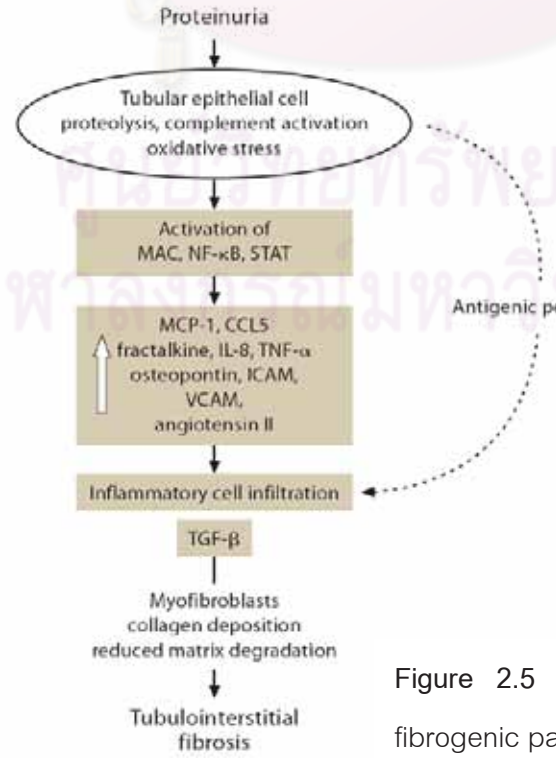


Figure 2.5 Activation of inflammatory and fibrogenic pathways in PTC by proteinuria (47)

Finally, striking feature observed during CKD is glomerulosclerosis, tubular atrophy and interstitial fibrosis (TA/IF) which correlate with progressive loss of renal function. TA/IF is a hallmark of chronic progressive kidney disease and is thought to be the final common mechanism that leads to ESRD. The pathology of CKD is characterized by relentless production and deposition of extracellular matrix (ECM) proteins, such as fibronectin and collagens within the interstitium of the kidney, and strongly correlates with deterioration of renal function (11, 54).

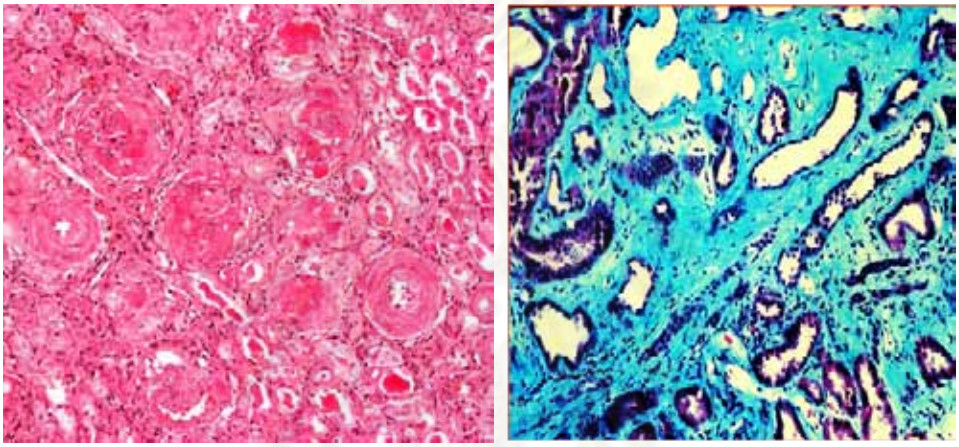


Figure 2.6 Histopathology in CKD patients demonstrate glomerulosclerosis, tubular atrophy and interstitial fibrosis

A better understanding of the pathogenesis of CKD leading to prevention and effective therapy is critical, such as strategies that suppress elaboration of ECM and thereby inhibiting the pathogenesis of tubulointerstitial fibrosis to prevent development of ESRD.

2.4 Mesenchymal-to-Epithelial (MET) Conversion

During embryonic development, the structures of the nephron from the glomerulus to distal tubule derive from the metanephric mesenchyme. The mesenchymal cells change their cell type and produce highly organized epithelia under the influence of signals from the ureteric bud. The morphological sequence of this conversion includes the formation of a corona of mesenchymal cells surrounding the tips of the ureteric bud, followed by the development of a pre-tubular aggregate, and then comma shape bodies, S-shaped bodies, eventually the glomerulus and the tubules, which evolves into preliminary forms of the segmented nephron (55, 56). Currently, these stages are largely based on histomorphologic criteria and expression of marker molecules. Bone Morphogenetic Protein-7 (BMP) family members display dynamic expression patterns during kidney development. BMP-7 transcripts are detected in the ureteric bud emerging from the Wolffian duct at 11.0 dpc and expression is maintained in derivatives of the bud throughout development. From the initial contact until the cessation of nephrogenesis, the condensed mesenchyme surrounding the ureteric tips expresses BMP-7 exclusively. Subsequently, the induced mesenchyme undergoes a process of nephrogenesis that involves several morphologically distinct stages proceeding from pre-tubular aggregate, comma and S-shaped tubules, to nephrons which are fused with the collecting duct at the distal end and contain a glomerulus at the proximal end. BMP family members are expressed in a graded manner during this period. BMP-2 expression in the kidney is first detected in the pretubular aggregates and is maintained in the distal part of the early tubules. By contrast, BMP-7 is expressed uniformly throughout the aggregates and tubules. Expression of both BMP-2 and BMP-7 is downregulated in the distal part as the tubule matures, though BMP-7 expression is maintained in the proximal part. In more developed tubules, BMP-3, BMP-4 and BMP-7 are all co-expressed in Bowman's capsule of the developing glomerulus (57).

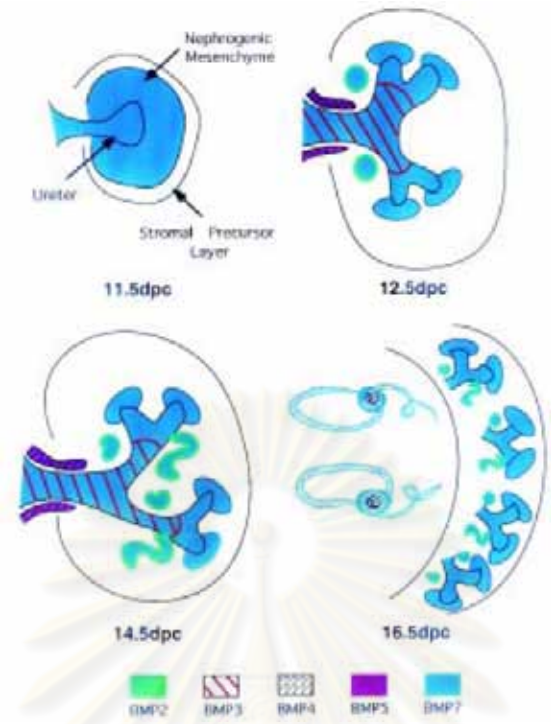


Figure 2.7 BMP family members are expressed throughout kidney development (57)

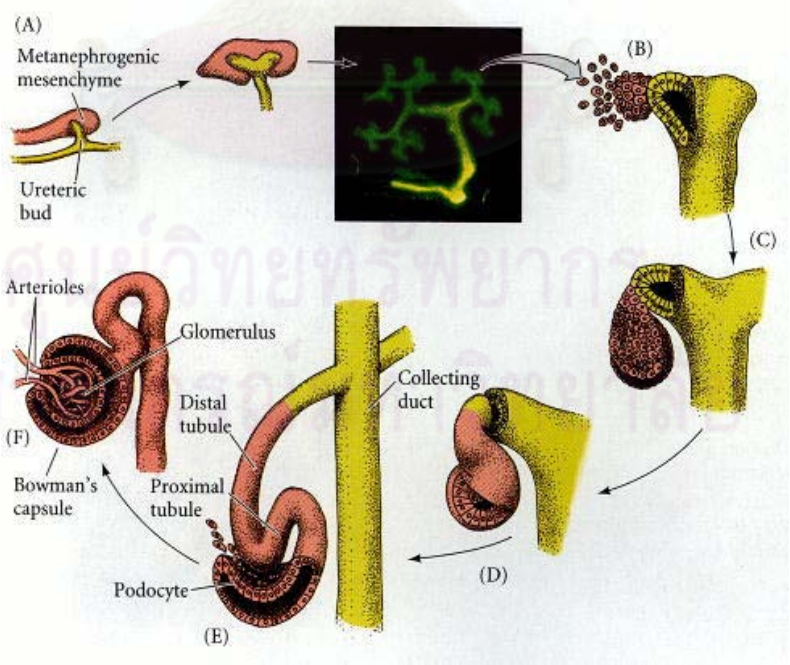


Figure 2.8 Normal kidney development and nephrogenesis (56)

BMP-7 and transforming growth factor β (TGF-β) regulate and maintain the epithelial state. Much of the renal epithelia are derived from mesenchymal cells through inductive interactions. One function of TGF-β might be to promote dedifferentiation or epithelial-to-mesenchyme transition (EMT), such that epithelial cells regress to a more mesenchymal phenotype. BMP-7 appears to counteract this by promoting the conversion of mesenchyme to epithelial cells or mesenchyme-to-epithelial transition (MET) (58).

2.5 Epithelial-to-Mesenchymal Transition (EMT)

Since late 20th century, the current thinking regards morphogenesis in early embryonic development, tissue repair, and cancer metastasis (59, 60). In mammals, experimental work on epithelial cell plasticity mainly follows the trail of two broad interests, metaplasia and epithelial-mesenchymal transition (EMT). With regard to EMT, it is important to note that it was agreed in 2003, at the first meeting of The EMT International Association (TEMTIA), in Port Douglas, Australia, that epithelial-mesenchymal transformation and epithelial-mesenchymal transdifferentiation would be called "**epithelial-mesenchymal transition**" going forward.

The field of EMT today is vastly more expansive in scope and understanding than it was just a few years ago, particularly with new work on the role of EMT in tissue fibrosis and cancer metastasis (59-61). The study of various model systems involving an abundance of different epithelial cell types, often examined in culture and out of biological context, lends considerable uncertainty to the nature of common signaling and transcriptional pathways predictive of EMT. This is particularly true when one tries to compare mRNA pools generated under various experimental conditions. In 2008, EMT meeting suggested there is heuristic value in parsing EMT into three general subtypes based simply on the context under which they occur. Type 1 EMT involves primitive epithelial cells transitioning to motile mesenchymal cells as part of gastrulation and primitive neuroepithelial cells generating migrating neural crest cells. In both situations, some of the cells generated by EMT are re-induced as secondary epithelial cells in mesodermal and endodermal organs by mesenchymal-epithelial transition (MET). Type 2 EMT involves secondary epithelial or endothelial cells transitioning to resident tissue

fibroblasts. In mature tissues, these fibroblasts are induced in response to persistent inflammation. Type 3 EMT involves epithelial carcinoma cells in primary nodules transitioning to metastatic tumor cells in order to migrate through the blood stream and, in some cases, form secondary nodules in distant metastatic sites by MET (62).

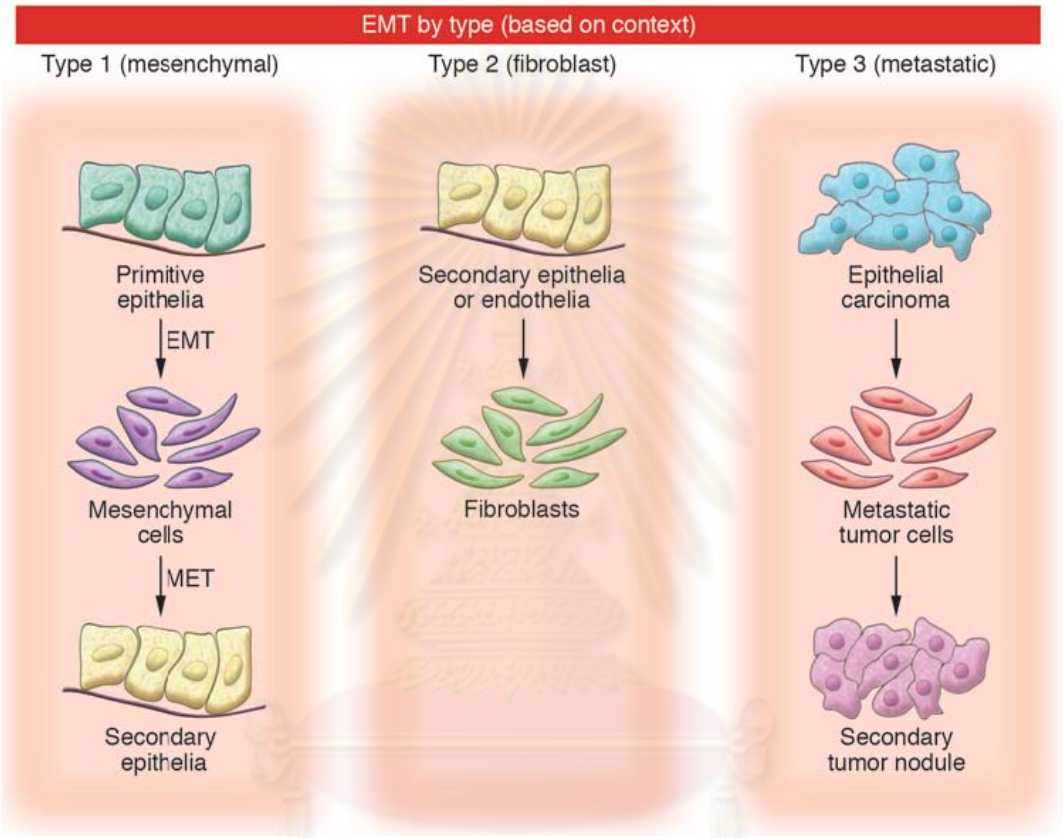


Figure 2.9 Three types of EMT are recognized depending on the phenotype of the output cells (62)

2.6 Biomarkers of EMT

A variety of biomarkers have been used to demonstrate all three subtypes of EMT. Here we examine a few of the more common markers, some of which are acquired and some of which are attenuated during transition.

2.6.1 Cell-surface markers of EMT: A change in expression of E-cadherin is the prototypical epithelial cell marker of EMT. E-cadherin is expressed in epithelial cells, and its expression is decreased during EMT in embryonic development, tissue fibrosis, and cancer (63). Moreover, loss of E-cadherin function promotes EMT (64). In addition, because OB-cadherin is a more definitive marker for activated fibroblasts, an E-cadherin–OB-cadherin switch is of interest for type 2 EMT associated with fibrogenesis (65).

2.6.2 Cytoskeletal markers of EMT: FSP1 is a member of the family of Ca2+-binding S100 proteins (66). It is a prototypical fibroblast marker for detecting EMT in cancer and fibrogenesis. In tissue fibrosis, most epithelial cells undergoing type 2 EMT express FSP1 early in transition to fibroblasts, and lineage tagging in transgenic reporter mice reveals that more than one-third of all FSP1+ fibroblasts in fibrotic kidneys are EMT derived (50). Alpha-SMA is one of six actin family members. In the adult, prominent alpha-SMA expression can be found in vascular smooth muscle cells and myoepithelial cells (67). Type 2 EMT, which contributes to tissue fibrosis, is also sometimes associated with cells that eventually express alpha-SMA as myofibroblasts.

2.6.3 Extracellular proteins: Fibronectin is a high-molecular weight glycoprotein that serves as a scaffold for fibrillar ECM (68). Even though fibronectin is an integral constituent of the fibrotic ECM associated with tissue fibrosis and the desmoplastic stroma in tumors, the utility of fibronectin as a type 2 and type 3 EMT biomarker is limited, in part, because it is produced by various cell types, including fibroblasts, mononuclear cells, and epithelial cells.

2.7 Epithelial-to-Mesenchymal Transition (EMT) in kidney fibrosis

Tubular atrophy/interstitial fibrosis (TA/IF) is a major cause of chronic progression loss of renal function in CKD. It is a chronic, progressive, nonspecific, and irreversible histopathologic entity, and is associated with significant CKD patient morbidity and mortality. Interstitial fibroblasts are the principal source of kidney fibrosis (50, 69). Under stress, interstitium fibroblasts expand by cell division and generate profibrotic molecules. Up to one third of all disease-related fibroblasts can originate from tubular epithelia at the site of injury through epithelial-to-mesenchymal transition (EMT) (50).

Tubular EMT by definition is a process in which renal tubular cells lose their epithelial phenotype and acquire new characteristic features of mesenchyme. Obviously, this phenotypic conversion is proposed as an orchestrated, highly regulated process that consists of four key steps: (*1*) loss of epithelial cell adhesion; (*2*) *de novo* _-smooth muscle actin expression and actin reorganization; (*3*) disruption of tubular basement membrane; and (*4*) enhanced cell migration and invasion. Of the many factors that regulate EMT in different ways, TGF-β is the most potent inducer that is capable of initiating and completing the entire EMT course, whereas and BMP-7 act as EMT inhibitors (13, 14).

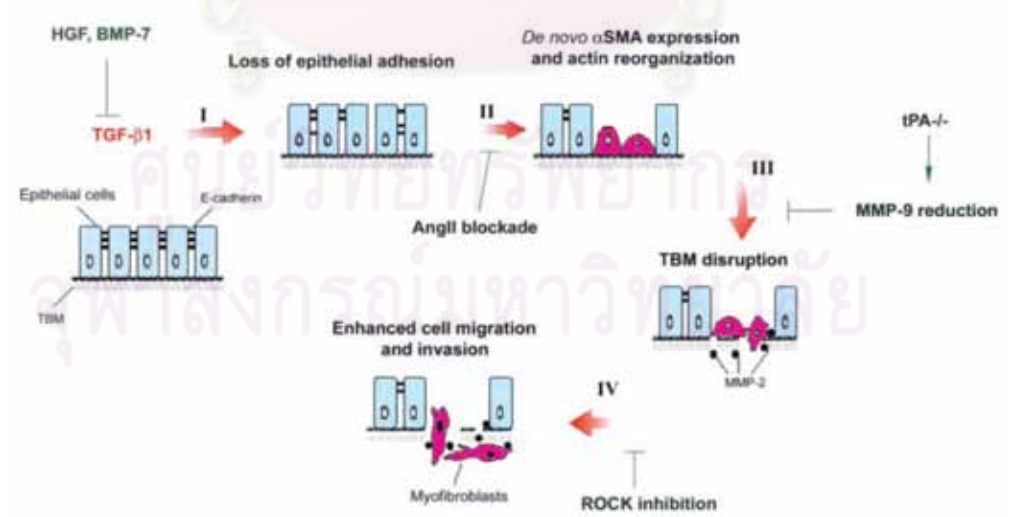


Figure 2.10 Schematic illustration shows the key events during tubular EMT (13)

2.8 TGF-β and BMP-7 induced Smad signaling pathway during EMT

Kidney fibrosis is an inevitable outcome of all kinds of progressive chronic kidney disease (CKD) (70). Despite a great deal of intense study, comprehensive understanding of the pathogenesis of renal scar formation after injury remains a daunting task that poses a major obstacle toward designing effective therapeutic strategies. In the past several years, epithelial-to-mesenchymal transition (EMT), a process by which fully differentiated epithelial cells undergo transition to a fibroblast phenotype, has emerged as an important pathway leading to generation of matrix-producing fibroblasts and myofibroblasts in diseased kidney. Many studies from different laboratories illustrate that tubular epithelial cells in vitro undergo phenotypic conversion after incubation with fibrogenic TGF-β; the transition is characterized by loss of epithelial proteins such as E-cadherin, zonula occludens -1 (ZO-1) and cytokeratin, and acquisition of new mesenchymal markers including vimentin, alpha-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP-1), interstitial matrix components type I collagen, and fibronectin (13, 71). These alterations in protein expression are usually accompanied by morphologic changes to a fibroblastoid appearance and an enhanced migratory capacity. Many studies proposed that EMT is an orchestrated, highly regulated process that consists of four key steps: loss of epithelial cell adhesion, de novo mesenchymal expression and actin reorganization, disruption of tubular basement membrane, and enhanced cell migration and invasion (13, 72).

The induction of EMT by TGF-β was first recognized in cell culture. Upon TGF-β treatment, epithelial cells changed from cuboidal to an elongated spindle shape, and showed decreased expression of epithelial markers and enhanced expression of mesenchymal markers fibronectin and vimentin (73). Mounting evidence establishes a crucial role for TGF-β signaling in mediating EMT (74, 75). TGF-β is the prototypic inducer of tubular and podocyte EMT (72, 76) whereas the effects of other mediators are often context-dependent, variable, and incomplete. Given the universal upregulation of its expression in the fibrotic kidney, TGF-β induced EMT is particularly relevant to the pathogenesis of kidney fibrosis. Smad proteins mainly mediate the signals of TGF-β. Upon stimulation by TGF-β, transmembrane type II TGF-β receptor forms tight complexes with the type I receptor, leading to phosphorylation and activation of Smad2

and Smad3. Phosphorylated Smads then heteroligomerize with the common partner Smad4 and translocate into the nucleus, where they control the transcription of TGF-β responsive genes through interaction with specific cis-acting elements in the regulatory regions (77, 78). The necessity of Smad signaling in EMT is clearly illustrated in vivo in Smad3 knockout mice after obstructive injury. Mice lacking Smad3 are protected from renal interstitial fibrosis and show reduced EMT and collagen accumulation after unilateral ureteral obstruction (79). Consistent with this, primary tubular epithelial cells from the Smad3 null mice are resistant to induction of EMT and key EMT regulatory genes (79, 80). Blockade of Smad signaling is also mechanistically linked to the inhibition of EMT by hepatocyte growth factor and bone morphogenic protein-7 (BMP-7) (16, 81).

Multiple BMPs are expressed in the kidney, of which BMP-7 is the most abundant and well characterized. BMP-7 appears to act as a survival factor for undifferentiated kidney mesenchymal cells by opposing apoptotic signals, and ensures that these cells are competent to respond to the inductive signals for promoting nephron formation (82). Several studies suggest that BMP-7 might have anti-inflammatory and cytoprotective effects on renal tubular epithelial cells. BMP-7 can suppress several TNF-alpha stimulated proinflammatory cytokines: interleukins 6 and -8, chemokines and monocyte chemoattractant protein-1. In addition, BMP-7 reduces the nuclear accumulation of Smad3 via a Smad5 mediated process. BMP-7 signaling via Smad5 upregulates Smad6, which blocks the nuclear translocation of phosphorylated Smads 2 and 3 and therefore counteracts TGF-β stimulated expression of plasminogen activator inhibitor-1 in mesangial cells (17). The opposing interactions of BMP7-dependent Smads (1 and 5) to the TGF-β induced Smads (2 and 3) might thus suppress TGF-β induced EMT (16). Thus, BMP-7 could restore the homeostatic balance of Smad signaling by promote Smad 1/5/8, preventing or reversing the development of EMT (83).

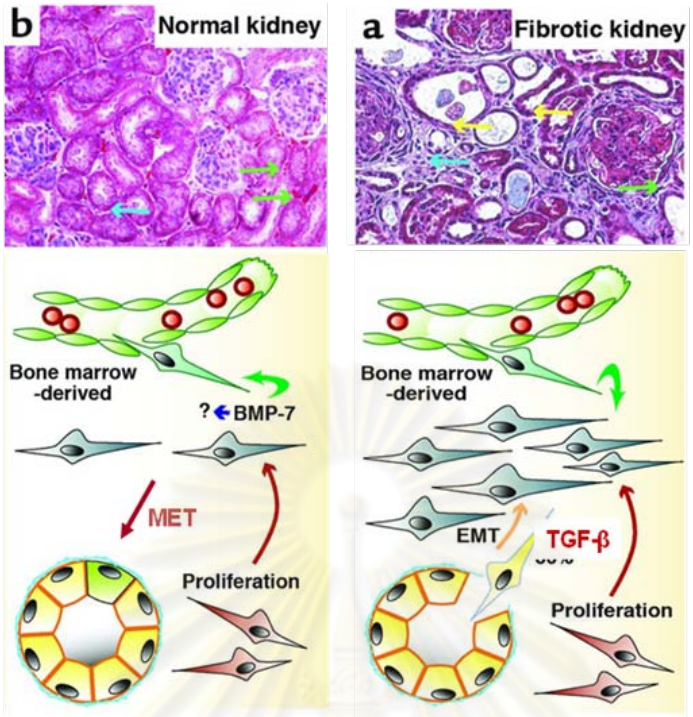


Figure 2.11 Counter balance of BMP-7 during MET and TGF-β during EMT

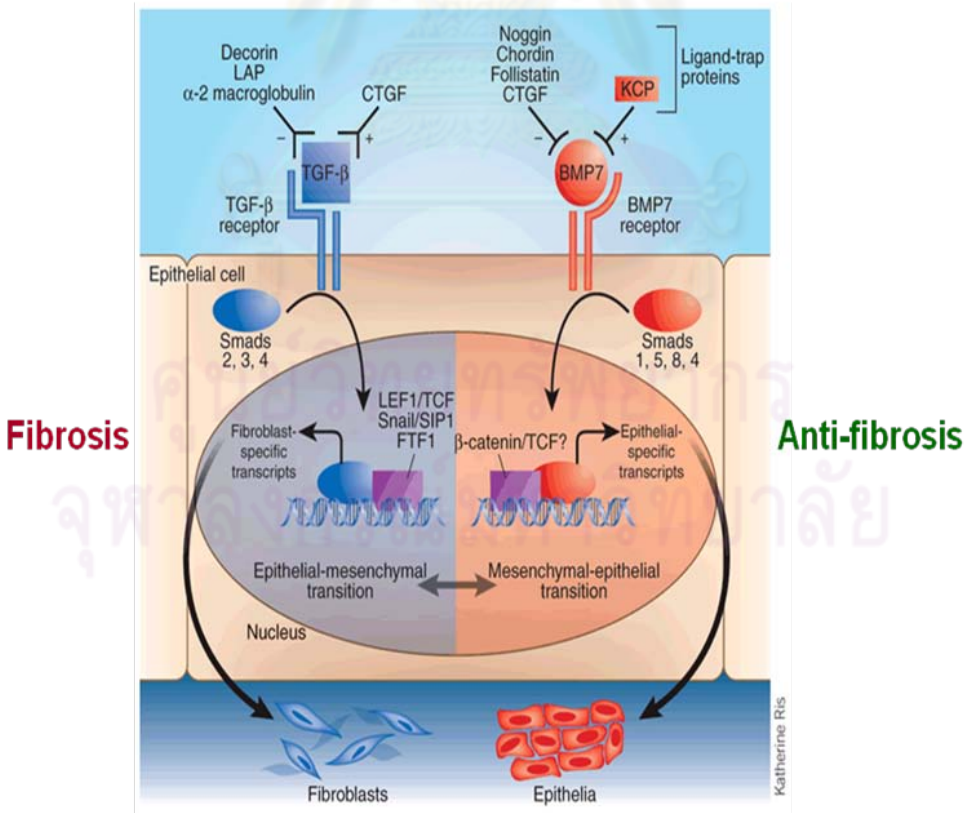


Figure 2.12 EMT controlled by TGF-β and BMP-7 induced Smad signaling pathway (15)

So, the therapeutic that can inhibit TGF-β/Smad2/3 and promote BMP-7 /Smad1/5/8 signaling could be the excellent treatment to attenuated EMT and renal fibrosis.

2.9 Vitamin E

Vitamin E is the name given to a family of eight molecules. They consist of two groups, tocopherols and tocotrienols, which contain saturated or unsaturated side chains, respectively. Vitamin E comprises 8 different forms, namely alpha-, beta-, gamma-, and delta-tocopherol and alpha-, beta-, gamma-, and delta-tocotrienol (84) and are differences in the bioavailability and bioequivalence which can lead to varying effects.

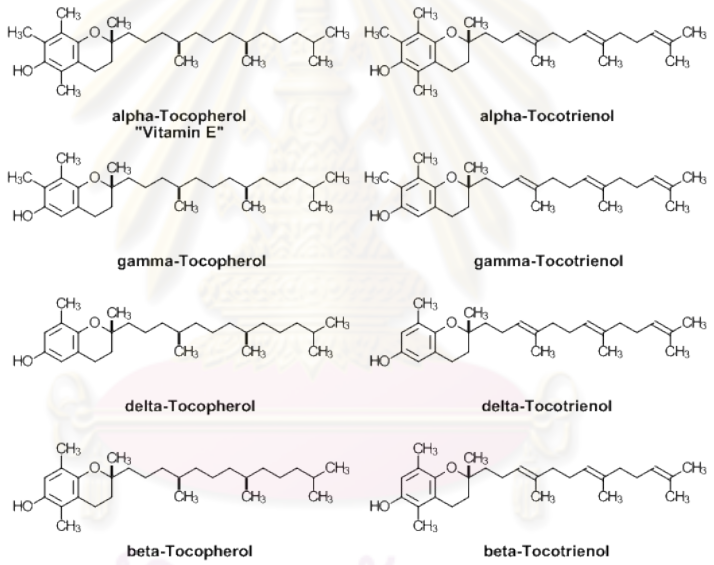


Figure 2.13 Eight different forms of the vitamin E

Vitamin E, particularly in the form of alpha tocopherol, has been proposed for the prevention or treatment of numerous health problems (85), which is primarily due to its antioxidant and anti-inflammatory properties (21, 86). Many studies indicate that the different vitamin E homologues also have biological activity unrelated to their antioxidant activity. Vitamin E's antioxidant function is that of a peroxyl radical scavenger that terminates chain reactions of oxidation of polyunsaturated fatty acids (PUFAs) (18). When lipid hydroperoxides (ROOH) are oxidized to peroxyl radicals (ROO·), as could

occur in the presence of free metals such as iron or copper, the ROO· react faster with α-tocopherol (Vit E-OH) than with PUFAs (87).

In the presence of vitamin E:

$$ROO\cdot + \text{Vit E-OH} \longrightarrow ROOH + \text{Vit E-O}\cdot$$

In the absence of vitamin E:

$$ROO\cdot + RH \longrightarrow ROOH + R\cdot$$

$$R\cdot + O2 \longrightarrow ROO\cdot$$

In this way, alpha tocopherol acts as a chain breaking antioxidant, preventing the further auto-oxidation of PUFAs in membranes or lipoproteins. Several studies have reported that vitamin E supplements are associated with decreased risk of various chronic diseases. The Women's Health Study, a ten-year prevention trial in normal, healthy women 45 years and older, found that 600 IU vitamin E taken every other day significantly decreased cardiovascular mortality by 24% and in women over 65 by 49% (20). The Cache County Study reported that antioxidant use (vitamin E >400 IU and vitamin C >500 mg) was associated with reduced Alzheimer disease prevalence and incidence in the elderly (88). These reports of beneficial vitamin E effects encourage the use of vitamin E supplements need for furthering understanding of vitamin E metabolism.

Supplement with vitamin E exhibit anti-inflammatory activity in both vitro and in vivo (84, 86). Alpha tocopherol was demonstrated to modulate two major signal transduction pathways centered on protein kinase C and phosphatidylinositol 3-kinase, which associated with changes in cell proliferation, platelet aggregation, and NADPH-oxidase activation. Human skin fibroblasts exhibit an age-dependent increase of collagenase expression that can be diminished by alpha tocopherol via protein kinase C inhibition (89). In smooth muscle cells and monocytes/macrophages, the oxidized LDL scavenger receptors SR-A and CD36 are transcriptionally down-regulated by alpha tocopherol but not by beta tocopherol (90, 91). Recently, the connective tissue growth factor transcription has been also found to be under the positive control of alpha tocopherol. Monocytes and neutrophils enriched with alpha tocopherol decrease their

adhesiveness both in vivo and in vitro (92, 93) due to the down-regulation of adhesion molecule expression (94). Alpha tocopherol inhibits aggregation of human platelets by a PKC-dependent mechanism, both in vitro and in vivo (95), and delays intra-arterial thrombus formation (96).

Oxidative stress	Inflammation/thrombosis
↓ LDL oxidative susceptibility	↓ hs-CRP
↓ Autoantibodies to ox-LDL	↓ Pro-inflammatory cytokines (IL-1 & 6, TNF)
↓ Urinary isoprostanes	↓ Monocyte adhesion to endothelium
↓ ROS (O_2^-) production in monocytes	↓ Soluble cell adhesion molecules
	↓ PAI-1
	↓ PGE_2 synthesis
	↓ Platelet aggregation

Abbreviations: hs-CRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; ox-LDL, oxidative low-density lipoprotein; PAI-1, plasminogen activator inhibitor-1; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; TNF, tumor necrosis factor; IL, interleukin.

Figure 2.14 Effect of vitamin E on oxidative stress and inflammation molecular function (21)

One of the most important key mediators that control inflammatory process is TGF-β, which has been shown down regulation of this gene expression by vitamin E (97, 98). Many studies in animal model show decreased expression of TGF-β and ameliorated renal injury by vitamin E treatment, example in pulmonary fibrosis (99), heart fibrosis (100), and chronic pancreatitis (101). In addition, vitamin E was demonstrated to suppress pro-fibrotic gene in some chronic renal injury model and diminish progression of renal fibrosis (19, 22). Moreover, vitamin E showed the benefit in many human diseases by decreased monocyte activity, soluble cell adhesion molecules (102), C-reactive protein and monocyte interleukin-6 (103) in diabetic patients. These evidences of vitamin E could be use an adjunctive therapy in the prevention of chronic inflammatory progression of atherosclerosis, atrophy and fibrosis in various organs.

Table 2.6 Modulation of gene expression by natural vitamin E (98)

GENE	PATHWAY	CELL LINE/TISSUE	EFFECT
CD36		smooth muscle cells, monocytes/macrophages	↓ αT
SR-BI		monocytes/macrophages	↓ αT
SR-AI/II		monocytes/macrophages	↓ αT
Tropomyosin		smooth muscle cells	↑ αT
Collagen α1(1)	ARE	liver stellate cells	↓ αT
MMP-1	PKC	fibroblasts	↓ αT
MMP-19	PKC	PBMC, HL-60	↓ αT
E-selectin	NF-κB	human endothelial cells	↓ αT
VCAM-1		THP-1 monocytes	↓ αT
ICAM-1		keratinocytes, neutrophils, endothelial cells, monocytes	↓ αT
Integrins		human erythroleukemia cells (HEL)	↓ αT
Glycoprotein IIb	PKC	platelets	↓ αT
CTGF	TGF-β-RE	smooth muscle cells, fibroblasts	↑ αT
Il-2		mouse T cells	↑ αT
IL-4	NF-κB, AP-1	human T cells	↓ αT
IL-1β		THP-1 monocytes	↓ αT
TGF-β		rat liver	↓ αT

In present study, the author will try to demonstrate the efficacy of reno-protective effect by vitamin E to slow progression of CKD. The advantage of vitamin E could inhibit the development of TA/IF in fibrotic kidney. Thus, the author hopes that amelioration of chronic kidney injury by vitamin E could be the alternative therapeutic approaches to the clinical treatment in CKD patients.

CHAPTER III

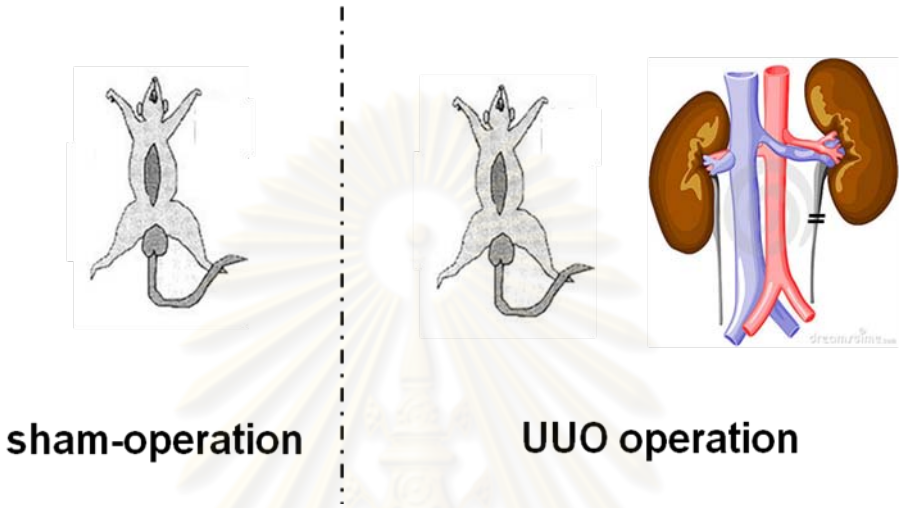
MATERIALS AND METHODS

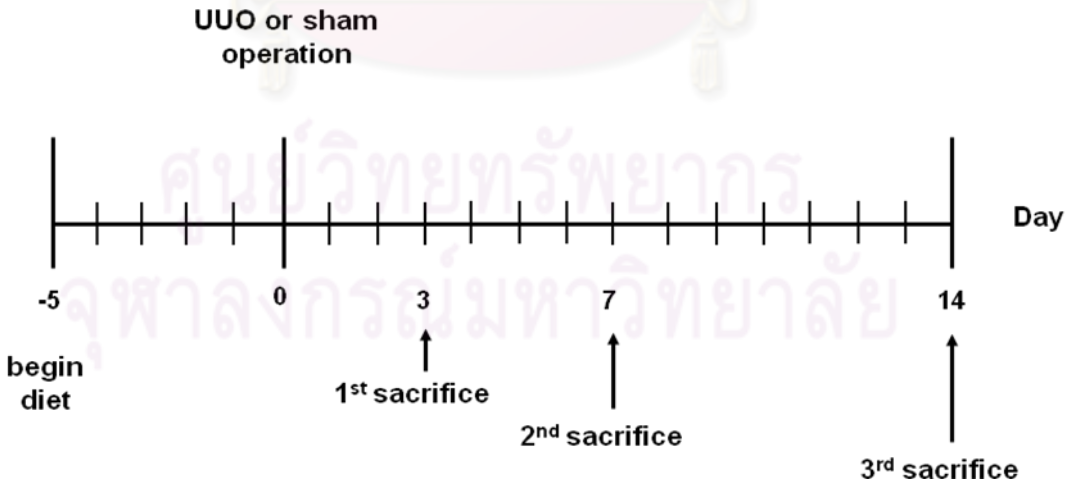
3.1 Animals care and experimental model

An official ethic committee in Thammasat University approved all experiments on animals. Male ICR mice weighing 25–30 g were obtained from National Laboratory Animal Center (Mahidal University) and allowed to acclimatise for 2 weeks prior to surgery. All mice received tap water and a standard diet and were housed in 12 hr light and 12 hr dark cycle.

All animal experiments were conducted in accord with the Thammasat Animal Experimental Unit Guideline. Mice were anesthetized with pentobarbital sodium at dose of 40–60 mg/kg by intra-peritoneal injections. The abdominal region was shaved, and the animals were placed on a heating table to maintain them at constant body temperature at 37 ± 1°C while under anesthesia. The abdomen was soaked with Betadine, and sterile drapes were applied. A midline abdominal incision was made, and both kidneys and ureters were identified. The left ureter was dissected out and ligated with 4.0 silk at two points along its length. The wounds were closed in two layers with 4.0 silk and mice were allowed to recover. Following surgery the animals were returned to the cages, where they had free access to food and water. Mice will be divided into the following four experimental groups (total = 48): (1) Sham-operated control group (n=6): mice were subjected to the surgical procedures described above except for the ureter ligation and were received oral placebo. (2) Sham-operated control + vitamin E group (n=6): these sham-operated mice were received oral vitamin E 250mg/kgBW. (3) UUO group (n=18): mice were subjected to the surgical procedures described above and were received oral placebo. (4) UUO + vitamin E group (n=18): these UUO mice were administered oral vitamin E 250mg/kgBW. Vitamin E and placebo were administrated everyday since 5 days before procedure and continue to day 14 post operation. One-third of mice were killed on day 3, one-third on day 7 after UUO or Sham operation, and the others on day 14. Kidneys were dissected from mice and sliced from the corona. These sections were fixed in 10% formalin and processed for histology using standard

techniques. A small section of the kidney was frozen in liquid nitrogen stored at -70 °C for protein measurements by Western blot analysis, while another section was fixed in RNA*later* Stabilization Solution (Ambion, Inc.) for RT-PCR gene expression studies.





3.2 Renal histology and immunohistochemistry

Kidneys were dissected from mice and tissue slices were fixed in 10% formalin and processed for histology examination using standard techniques. Formalin tissue was embedded in paraffin and 4 micrometer sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and masson's trichrome. These sections were examined in a blinded fashion by a nephrologist. The percentage of histology changes, such as degree of glomerulosclerosis, tubular atrophy, and interstitial fibrosis were evaluated under high power magnification (400x) in 5 to 10 consecutive fields, and mean percentages of histological change were then calculated.

The kidneys were fixed in 4% paraformaldehyde. Five-micrometer paraffin sections were dewaxed and rehydrated. For antigen retrieval, kidney sections were microwaved for 30 minutes. Endogenous peroxidase was quenched with 3% H_2O_2 for 20 min, and non-specific binding blocked with 20% normal goat serum in phosphate-buffered saline (PBS) (pH 7.4). Sections were incubated at 4°C with primary antibodies against **TGF-β1** (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), **Smad2/3** (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), **BMP-7** (1:200; Abcam: Biomed Diagnostics (Thailand) Co. Ltd.), **S100A4** (1:200; Abcam: Biomed Diagnostics (Thailand) Co. Ltd.) for 1-3 hr followed by Envision reagent (Dako, Bangkok Thailand) containing anti-rabbit secondary antibodies for 30 min, and finally with 3,5-diaminobenzidine (DAB) substrate for 10 min. Negative controls using normal rabbit IgG were also included. Nuclei were counterstained with hematoxylin for 2 min, and slides were dehydrated and mounted with permount.

3.3 Standard protein techniques

3.3.1 Protein extraction

Briefly, 40 mg of kidney (wet weight) was homogenized in 240 µl of 40 mM Tris-HCl (pH 7.6) buffer containing 0.1% Nodinet P-40, 0.05% sodium deoxycholate, 0.01% SDS, 150 mM NaCl, and 10 mM 2-mercaptoethanol. Homogenates were treated with 60 µg/ml of PMSF and centrifuged in a pre-chilled rotor at 15,000xg for 15 min. Supernatants were stored at -70°C. Protein content was measured using a BCA™ Protein Assay Kit (PIERCE, IL, USA).

3.3.2 Determination of protein concentration

Protein concentrations were determined using BCA (Bicinchoninic acid) Microtitre Protein assay. In a 96 well microtitre plate, 20 µl of distilled water (in duplicate) was used as a blank. Varying amounts of a 1mg/ml of BSA protein standard was also loaded, in duplicate, such that each pair of standards contained between 2 and 20 µg. 5, 10 and 20 µl of unknown sample were added separately in duplicate wells. All standard proteins and samples were made up to 20 µl final volume with distilled water and 200 µl of standard-working reagent (S-WR) added. This working reagent was stable at room temperature for approximately 1 week. The plate was placed, covered with cling film to reduce evaporation, in an incubator for 30 min at 37 °C and then removed to cool at room temperature before measuring the absorbance at a wavelength of 600 nm using a plate reader (ELx 800 UV, Universal Microplate Reader; Bio-Tek Instruments, INC). A protein standard curve was created and the protein concentration of unknown samples was determined.

3.3.3 Standard denaturing Laemmli PAGE

Reagents needed as described in 2.2.12. The resolving gel (typically 10 ml per gel) was made up to the desired final percentage acrylamide containing resolving buffer (375 mM Tris-Cl, pH 6.8 and 0.1% SDS) according to the following table:

% resolving gel	7.5%	10%	12.5%	15%
30% acrylamide stock (ml)	2.5	3.33	4.16	5.00
Resolving buffer (ml)	2.51	2.51	2.51	2.51
Water (ml)	4.99	4.16	3.33	2.49
TEMED (µl)	10	10	10	10
10% AMPS (µl)	45	45	45	45

The gel solution was polymerized by addition of TEMED and AMPS (as indicated above), briefly mixed and immediately poured into a gel cassette. The resolving gel was overlaid with water saturated n-butanol and allowed to polymerize for at least 30 min. Prior to adding the stacking gel, the butanol was removed and the surface of the resolving gel was rinsed with distilled water. The stacking gel was then made as shown below with a final acrylamide concentration of 4 % in 5 ml. This was poured on top of the resolving gel and the comb placed into the stacking gel to allow well formation; the stacking gel was allowed 30 min to completely polymerise.

30% acrylamide stock (ml)	0.67
Stacking gel buffer (ml)	0.63
Water (ml)	3.70
TEMED (µl)	15
10%AMPS (µl)	30

The gel was placed in a suitable running tank filled with 1xrunning buffer. 3 volumes of sample were diluted by the addition of 1 volume of 4xsample loading buffer and heated for 10-15 min at 70 ºC before loading into wells. The gel was run at a constant voltage of 150 volts for approximately 1.15 h, until the dye front reached the bottom of the gel. The molecular weight standard markers were loaded (5 µl per well) in one well of each gel.

3.3.4 Western blotting

Proteins separated via PAGE gel were transferred to nitrocellulose membrane by using 1xTowbin's transfer buffer or 1xBolt and Mahoney's transfer buffer at 250 mA for 1-2 h. If non-prestained standard protein markers were used, the blotted membrane was stained with Ponceau S and the position of standards marked prior to blocking. The blotted membrane was blocked with 5% skimmed milk powder (SMP) or 5% bovine serum albumin (BSA) in TBS/Tween at room temperature with gentle shaking for 1 h. The blocked nitrocellulose membrane was either subsequently subjected to immunoblotting or stored at -20 ºC until use.

3.3.5 Western immunoblotting

The blocked membrane was incubated with primary antibody solution at the desired dilution in 1% SMP or 5% BSA in TBS/Tween overnight on a roller at 4 °C. After overnight incubation, the membrane was washed three times with TBS/Tween for 10 min each. The membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at the appropriate dilution in TBS/Tween for 1 h at room temperature after which the membrane was washed three times in TBS/Tween as previously and rinsed once with TBS.

ECL reagent (Pierce) was added to the surface of the membrane for 1 min and the excess ECL reagent was removed. The membrane was wrapped in cling film and placed face up in an X-ray cassette. In a dark room, a suitable piece of X-ray film was placed over the ECL incubated membrane for the appropriate exposure time and subsequently developed in developer solution and fixer solution, respectively, according to manufacturer's instructions.

The antibodies list below were used to detect their respective proteins after transfer using Towbin's buffer and blocking in 5% skimmed milk powder in TBS/Tween.

Primary antibody			Secondary antibody
Anti-TGF-β1	(1:10,000)	Santa Cruz	anti-rabbit (1:5000)
Anti-Smad2/3	(1:1,000)	Santa Cruz	anti-rabbit (1:5000)
Anti-Smad1/5/8	(1:1,000)	Santa Cruz	anti-rabbit (1:5000)

Anti-BMP-7	(1:500)	Abcam	anti-rabbit (1:5000)
Anti-S100A4	(1:1000)	Abcam	anti-rabbit (1:5000)
Anti-actin	(1:2000)	Santa Cruz	anti-rabbit (1:2500)

Other proteins were transferred to membrane with Bolt and Mahoney's transfer buffer. The blotted membrane was also blocked with 5% BSA containing 50 mM NaF in TBS/Tween. The primary antibody was diluted in 5% blocking buffer.

To detect His-tagged recombinant protein, the blotted membrane was blocked with 2.5% BSA in TBS/Tween at room temperature for 1 h. The blocked membrane was washed three times with TBS/Tween for 10 min each and incubated in India HisProbe solution at a 1:5,000 dilution in TBS/Tween for 1 h and subsequently processed with three washes. The membrane was then developed using ECL detection as described previously.

3.3.6 Western blot stripping

After ECL detection, if blots were to be re-probed, the blot was rinsed with TBST several times to remove ECL reagent and were then incubated in stripping buffer for 30 min at 55-60 ºC in a heat-sealed bag. Subsequently, the blot was washed three times with TBS/Tween for 10 min each and the membrane was blocked with 5% skimmed milk powder in TBS/Tween before immunoblotting.

3.3.7 Microwave staining of SDS-PAGE gels using colloidal coomassie G250 stains

Prior to staining, the gel was submerged in deionized water and heated in a microwave oven on high power for 1 min until the water began to boil followed by shaking on a rocking platform for 1 min; the water was then discarded. This washing step was repeated twice more to remove SDS from the gel. The gel was submerged in ammonium sulphate colloidal G250 stain and microwaved as before. The container was placed on a gently rocking platform to allow staining to continue until the necessary protein bands were visible in the gel. The stained gel was subsequently rinsed several times in distilled water with shaking to generate a clear background.

3.4 Real time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen, Chatworth, CA, USA) according to the manufacturers' instructions. High-quality RNA was eluted in 35 μl RNase-free water. An aliquot of each RNA preparation was used to determine total RNA quality and concentration, measured at 260 nm (OD_{260}). Pure RNA possessed an OD_{260}/OD_{280} ratio of 1.6-1.9. Total RNA (0.25 ug) was reverse-transcribed to cDNA by Taqman™ Reverse Transcriptase Reagent (Applied Biosystems, Roch Molecular Biochemical, NJ, USA) using random primers using the following cycling conditions: 25°C, 10 min; 48°C, 30 min; 95°C, 5 min. The mRNA levels of TGF-β1, Smad3, Smad8, BMP-7, and hypoxanthine phosphoribosyltransferase (HPRT) were measured using a ABI PRISM 7700 Sequence Detection System (SDS version 1.6; PE Applied Biosystems). The primers and probe used were as follows.

Table 3.1 Gene and sequence using in real time RT-PCR

Gene	Sequence
TGF-β1	forward:5'-GGCTACCATGCCAACCAGCCTGGTGTACTCA-3', reverse: 5'-CCGGGTTGTGTTGGTTGTAGA-3', probe: 5'-FAM-CACACAGTACAGCAAGGTCCTTGCCCT-TAMRA-3';
Smad3	forward: 5'-GGGCCTACTGTCCAATGTCA-3', reverse: 5'-CCCAATGTGTCGCCTTGTA-3', probe: 5'-FAM-CCGGAATGCAGCCGTGGAAC-TAMRA-3';
Smad8	forward: 5'-CCTATCAACACTCAGACTTCCG-3', reverse: 5'-GTGAAGCCGTCTATGAGCAC -3', probe: 5'-FAM-ACTTTCCAGGCGTCCTCGCG-TAMRA-3';

BMP-7	forward: 5'-TGGATGGGCAGAGCATCAA-3', reverse: 5'- CTTGGAG CGATTCTGGCTG-3', probe: 5'-ATTGGACGGCATGGACCCCAGA-3';
HPRT	forward: 5'-TGACACTGGTAAAACAATGCAAACT-3', reverse: 5'-AACAAAGTCTGGCCTGTATCCAA-3', probe: 5'-TTCACCAGCAAGCTTGCAACCTTAACC-3';

The probes were labelled with 6-carboxy-fluorescein (FAM) at the 5'end, and with 6-carboxytetramethylrodamine (TAMRA) at the 3'end. FAM serves as the reporter dye, and TAMRA serves as the quencher dye. All primer pairs were designed to span across intron-exon boundaries in order to test for any genomic DNA contamination of RNA samples. Each PCR was assembled in 20 μl volumes consisting of 10 μl of 2xQuantiTech Probe mastermix (Qiagen, Chatworth, CA, USA), 0.5 μl of 20μM forward primer, 0.5 μl of 20μM reverse primer, 0.2 μl of 20μM probe and 6.8 μl of RNase-free water. Following the addition of 2 μl of cDNA template, PCR amplification was performed using an initial denaturation step at 95°C for 15 minutes, then 50 cycles of heating at 95°C and immediate cooling to 58°C (for Ang-1) or 55 °C (for HPRT) for 60 seconds. Real-time PCR results were automatically recorded by ABI PRISM 7700 Sequence Detection System (SDS version 1.6; PE Applied Biosystems) and analyzed by relative quantification using the comparative Ct method. Ratios for TGF-β/HPRT, Smad3/HPRT, Smad8/HPRT, and BMP-7/HPRT mRNA were calculated for each sample and expressed as the mean ± s.d.

3.5 Statistical analyses:

Data were expressed as mean ± SD. Statistical analyses were carried out using the SPSS software (version 15.0). Statistically significant differences among groups were calculated by ANOVA Bonferroni and Mann-Whitney tests using the least significant difference method. Statistical significances were defined as $p<0.05$.

CHAPTER IV

RESULTS

4.1 Vitamin E protected against renal fibrosis in mice UUO model

The author assessed the renoprotective effect of vitamin E on histopathology lesion of obstructed kidney. From H&E and PAS staining, the UUO animals exhibited significant tubular dilation and atrophy since day 3 (Figure 4.1, B). The degrees of these changes were more severe in day 7 and day 14 (Figure 4.1, C and D) than day 3. By the way, control kidney showed normal histoarchitecture, with distinct cortex, medulla, and renal papilla (Figure 4.1, A). Interestingly, the degree of tubular dilation and atrophy was decreased with vitamin E treatment in any time course (Figure 4.1, F - H) when compared with placebo treatment. No pathologic changes were noted in the kidneys from sham-operated mice (Figure 4.1, E). From PAS staining, the UUO animals exhibited significant TA for 10% since day 3, 19% at day 7, and progressed to 45% at day 14 after UUO compared with the sham control ($P<0.05$) (Figure 4.2). However, the severity of TA was significantly lower in obstructed kidneys with vitamin E treatment, 4% at day 3, 7% at day 7, and 19% at day 14 compared with the placebo treatment ($P<0.05$) (Figure 4.2).

From Masson trichrome staining, the collagen deposit in interstitial area was detected since day 3 in obstructed kidney (Figure 4.3, B). The degree of interstitial fibrosis in obstructed kidney was more severe at day 7 and day 14 after undergoing UUO (Figure 4.3, C and D) than the control (Figure 4.3, A). In contrast, treatment with vitamin E suppressed collagen deposit in UUO mice any time course since day 3 through day 14 (Figure 4.3, F - H) compared with the sham treatment group (Figure 4.3, E). There was no pathological change in kidneys from sham-operated mice. Kidney sections demonstrated a significantly increased of collagen deposit in interstitial area for 10% since day 3, 20% at day 7 and growth to 59% at 14 days after undergoing UUO in placebo treatment ($P<0.05$) (Figure 4.4) compared with the sham control. In contrast, treatment with vitamin E significantly suppressed the changes of collagen deposit in UUO mice to only 4% at day 3, 12% at day 7, and 26% at day 14 compared with the placebo treatment ($P<0.05$) (Figure 4.4).

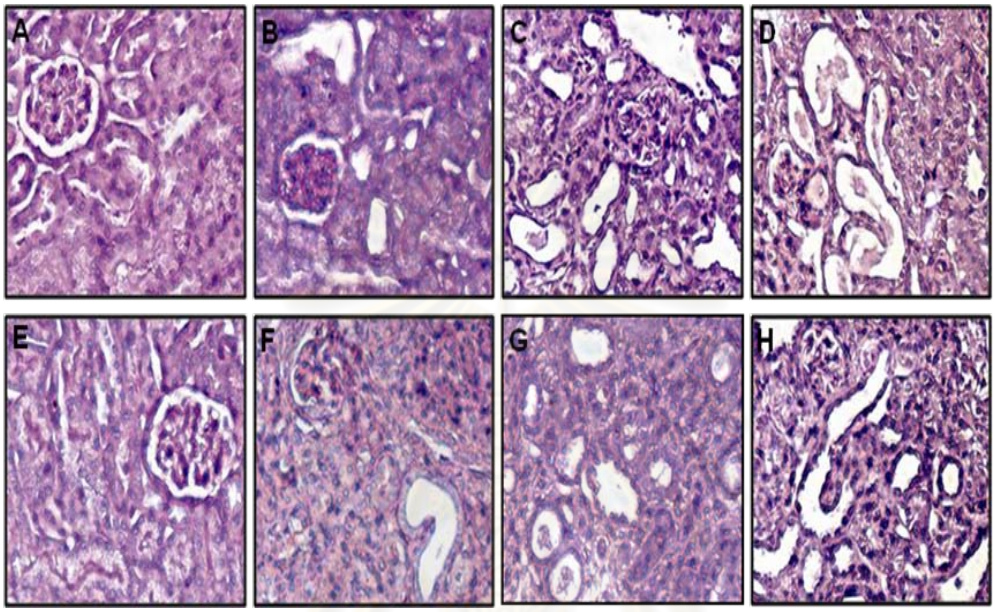


Figure 4.1 Treatment with vitamin E inhibited progression of tubular dilatation and atrophy in UUO mice. (A) sham-operated control showed normal histoarchitecture. The obstructed kidneys showed progressive tubular dilatation and atrophy at day 3 (B), day 7 (C), and day 14 (D) after UUO compared with the sham group which was apparently ameliorated by vitamin E treatment in any time course (F - H).

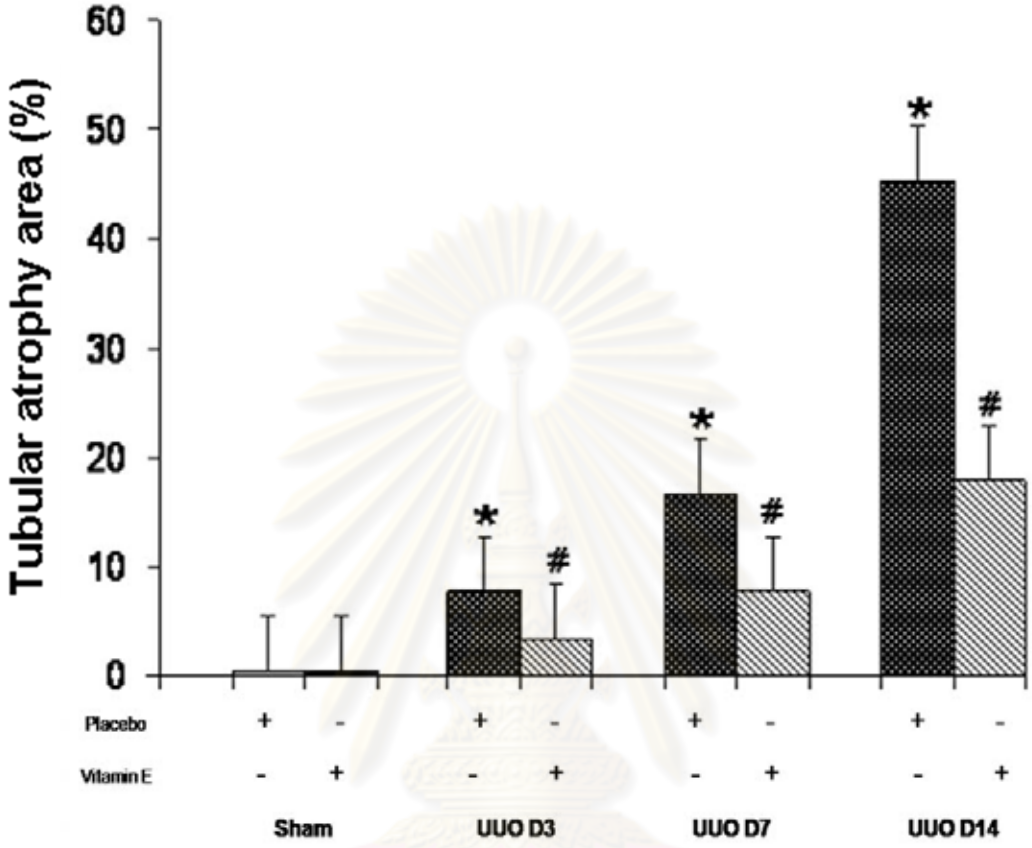


Figure 4.2 The percentage changes of tubular atrophy in UUO mice.

Significant difference $^{*}P < 0.05$ compared with sham group; $^{\#}P < 0.05$ compared with UUO with placebo treatment group

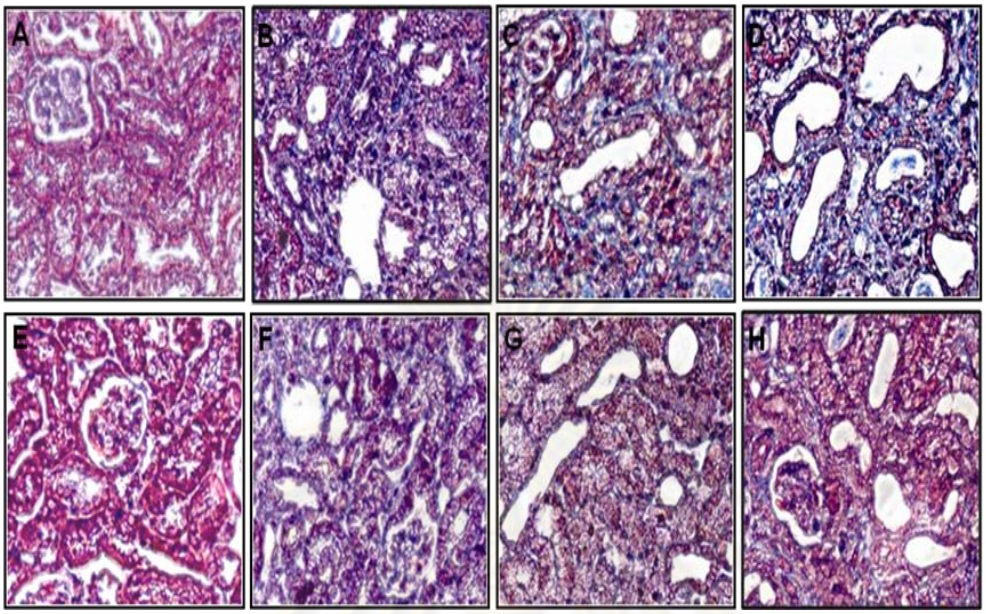


Figure 4.3 Treatment with vitamin E inhibited progression of interstitial fibrosis in UUO mice. (A) sham-operated control showed normal histoarchitecture. The obstructed kidneys showed progressive interstitial fibrosis at day 3 (B), day 7 (C), and day 14 (D) after UUO compared with the sham group. UUO mice with vitamin E treatment demonstrated the amelioration of interstitial fibrosis in any time course (F - H) compared with sham-operation treatment (E).

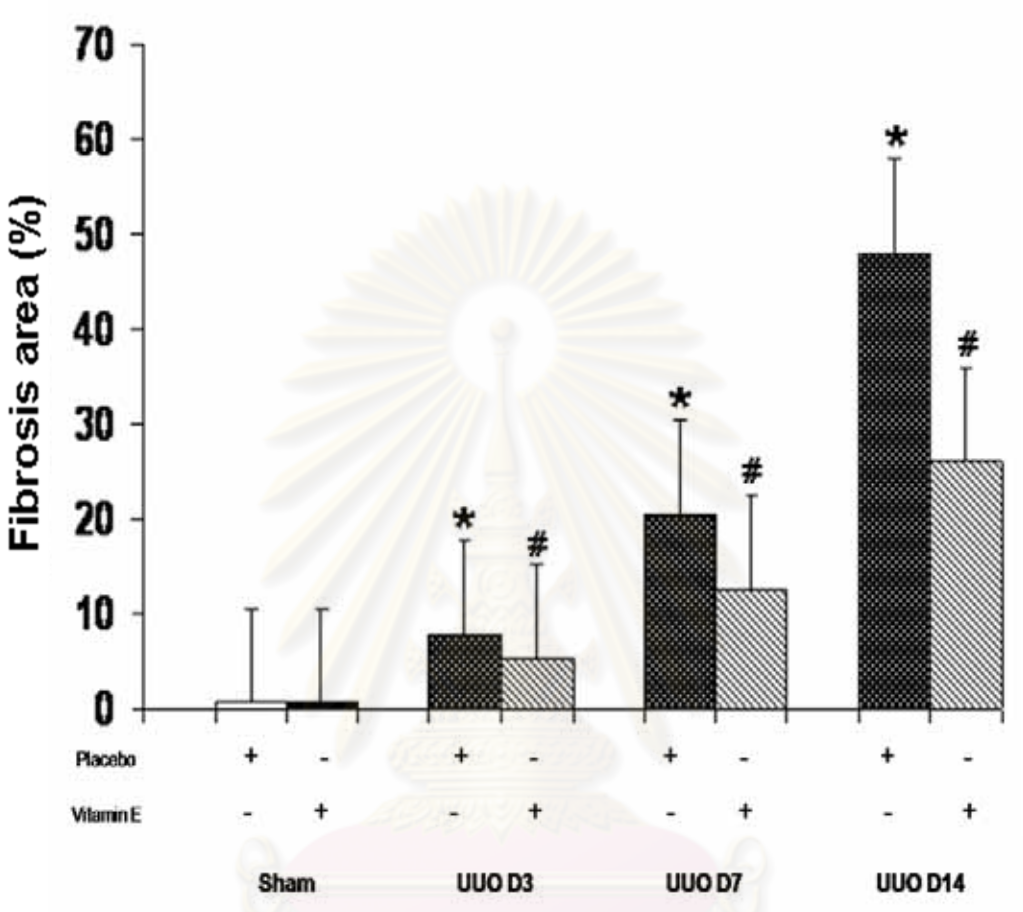


Figure 4.4 The percentage changes of interstitial fibrosis in UUO mice.

Significant difference $^{*}P < 0.05$ compared with sham group; $^{\#}P < 0.05$ compared with UUO with placebo treatment group

4.2 Vitamin E inhibited expression of extracellular matrix, and S100A4

The author attempted to clarify the localization and expression of S100A4 during the time course of UUO mice. The author found that S100A4 could not be detected in any tubule or interstitial area (Figure 4.5, A) in sham kidneys. However, S100A4 staining was detected in some TEC and cells in interstitial area that display the outline of lymphocytes within the fibrosing obstructed kidneys. Staining of S100A4 was increased at day 3, day 7 (Figure 4.5, B and C) and further increasing was observed at day 14 (Figure 4.5, D) compared with the sham control (Figure 4.5, A). In contrast, reducing of S100A4 staining was observed in the vitamin E treatment UUO kidneys (Figure 4.5, F - H), compared with placebo treated UUO mice during any time course. Higher quantity of S100A4 protein during obstruction process was also confirmed by using Western blot analysis (Figure 4.6). Consistent with immunohistochemistry, S100A4 protein level became significantly increased since day 3, day 7 and day 14 after undergoing UUO when compared to sham kidney ($P<0.05$) (Figure 4.6). However, UUO mice treated with vitamin E showed significantly decreased S100A4 protein level in any time course compared with the placebo treatment group ($P<0.05$) (Figure 4.6).

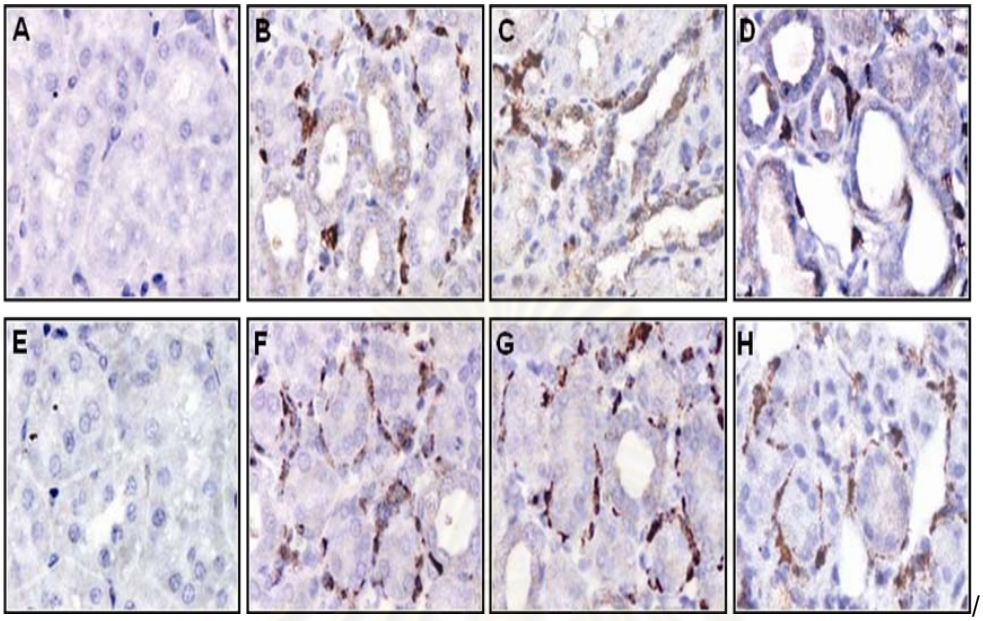


Figure 4.5 Immunohistochemical labeling of S100A4 in UUO mice. (A) In sham group, no S100A4 staining was detected in the kidneys. In obstructed kidney, increasing of S100A4 staining was seen on cells in interstitial area that display the outline of lymphocytes and some TEC within the fibrosing obstructed kidneys compared with the sham at day 3 (B), day 7 (C), and day 14 (D). However, decreasing of S100A4 expression was observed in UUO mice with vitamin E treatment at day 3 (F), day 7 (G), and day 14 (H) compared with placebo treatment groups.

S100A4 protein in any time course

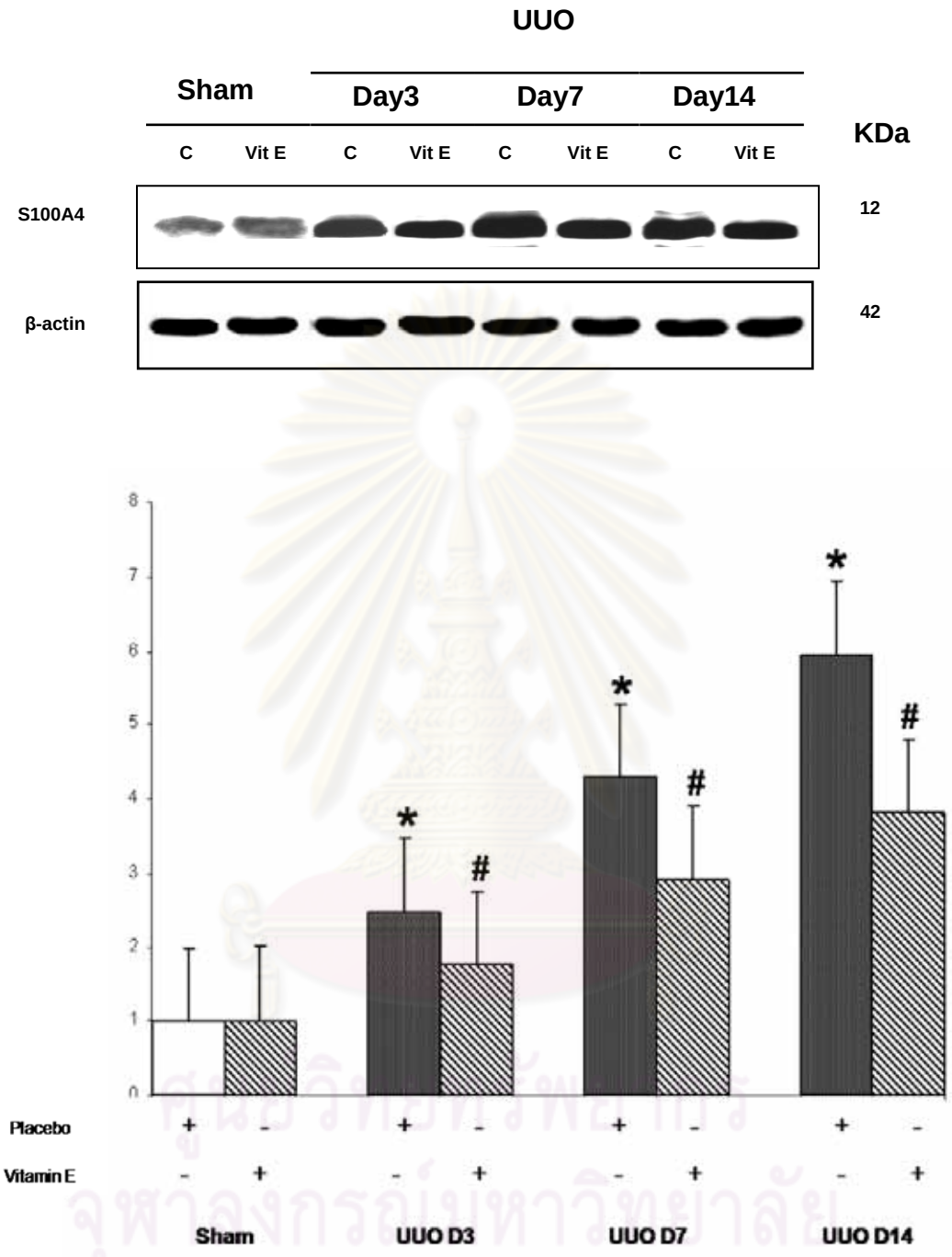


Figure 4.6 Western blot analysis for S100A4 protein expression in UUO mice. S100A4 protein expression was significantly increased in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. Treatment with vitamin E resulted in a decrease in the levels of S100A4. *$P < 0.05$ *vs* sham group; #$P < 0.05$ *vs* UUO with placebo treatment group.

4.3 Vitamin E treatment attenuated increased TGF-β1 expression in UUO kidneys

The author tried to investigate the effect of vitamin E could prevent renal fibrosis. The changes in the protein level and mRNA of TGF-β1 in the obstructed kidneys at day 3, day 7 and day 14 after UUO were observed. By immunohistochemistry, TGF-β1 revealed no labeling in sham kidneys (Figure 4.7, A), whereas the recognition of TGF-β1 was strongly detected at the interstitium area of placebo treated obstructed kidneys since day 3 after UUO (Figure 4.7, B) and further increased staining were demonstrated at day 7 and day 14 (Figure 4.7, C and D). Quantity assessment by Western blot analysis demonstrated progressive increased TGF-β1 protein level in the obstructed kidneys compared with the sham kidneys (Figure 4.8). In contrast, UUO mice treated with vitamin E was associated with decreased TGF-β1 staining during the time course of obstruction (Figure 4.7, E - H). In addition, TGF-β1 protein level revealed significantly decreased in the vitamin E treatment obstructed kidneys, compared with placebo treated group in any match time course ($P<0.05$) (Figure 4.8). Besides, sequence of TGF-β1 mRNA upregulation was demonstrated in time course of obstructed kidneys. However, treatment with vitamin E in UUO mice became significantly lower TGF-β1 mRNA expression compared with UUO with placebo treatment ($P<0.05$) (Figure 4.9).

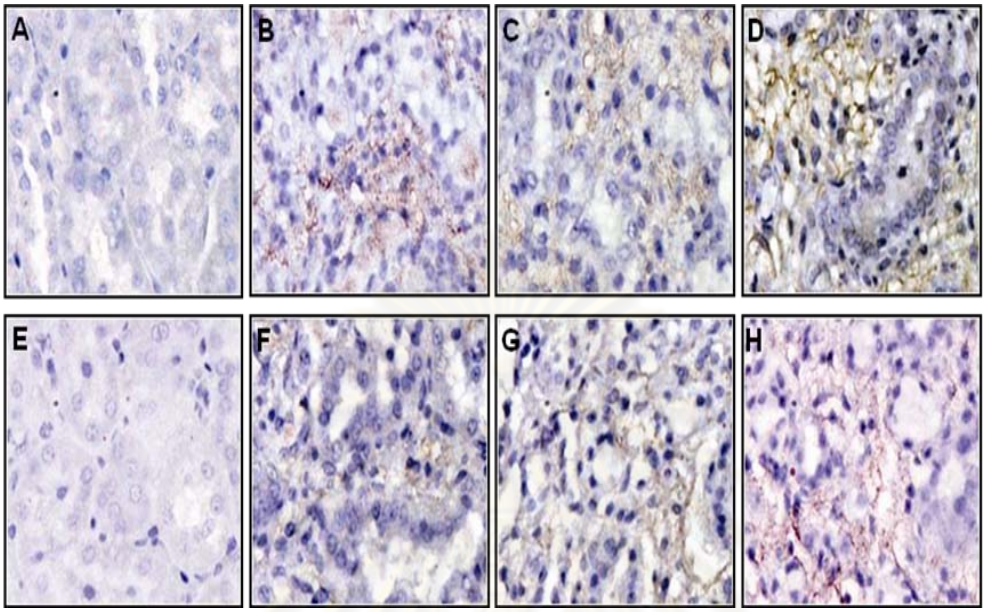


Figure 4.7 Immunohistochemical labeling of TGF-β1 in UUO mice. (A) In sham group, no TGF-β1 staining was detected in the kidneys. In obstructed kidney, advance increased TGF-β1 staining was seen in interstitial area within the fibrosing obstructed kidneys compared with the sham at day 3 (B), day 7 (C), and day 14 (D). However, decreased of TGF-β1 expression was observed in UUO mice with vitamin E treatment at day 3 (F), day 7 (G), and day 14 (H) compared with placebo treatment groups.

TGF-β1 protein in any time course

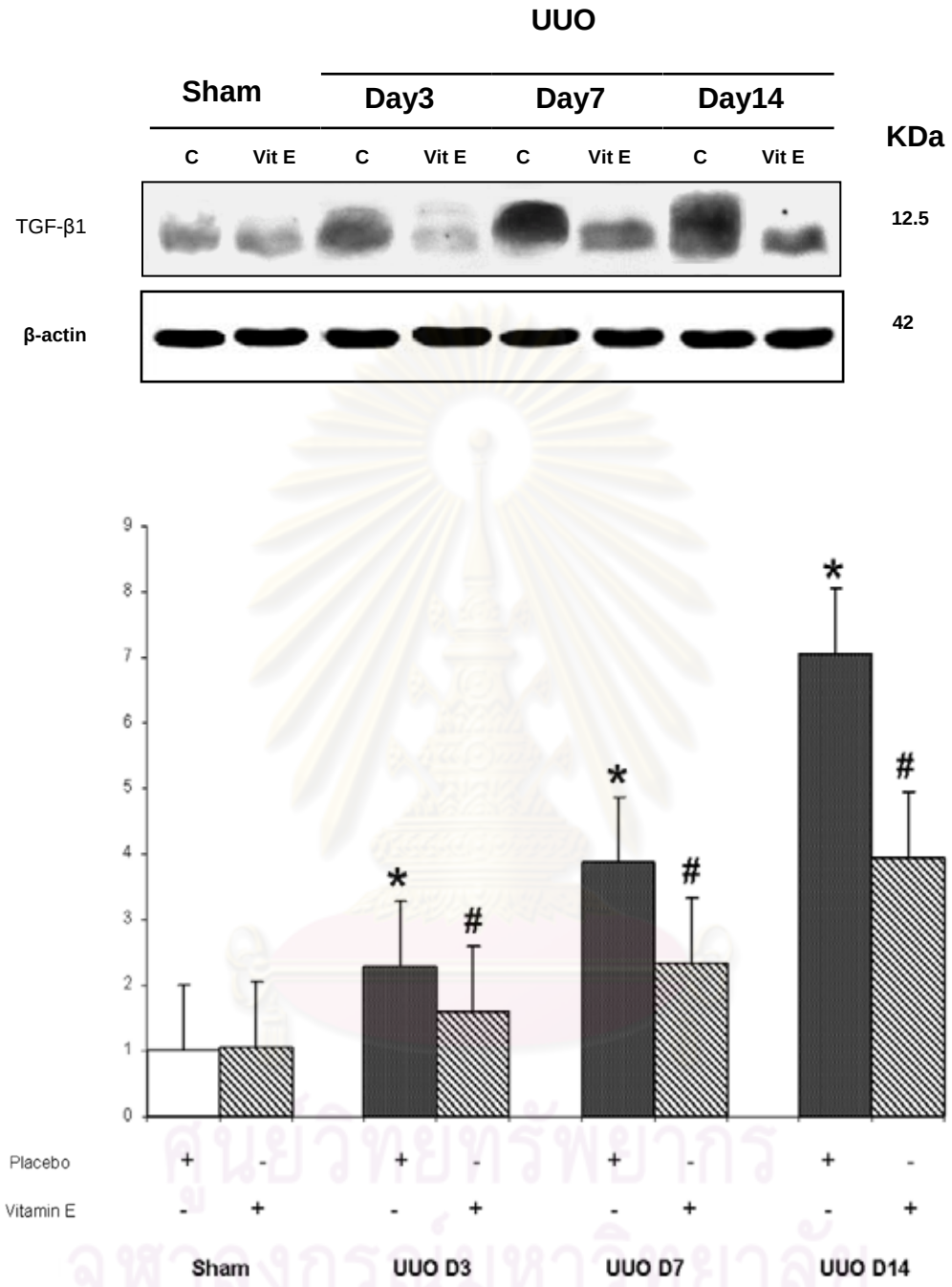


Figure 4.8 Western blot analysis for TGF-β1 protein expression in UUO mice. TGF-β1 protein expression was significantly increased in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. Treatment with vitamin E resulted in a decrease in the levels of TGF-β1. *$P < 0.05$ *vs* sham group; #$P < 0.05$ *vs* UUO with placebo treatment group.

TGF-β1 mRNA in any time course

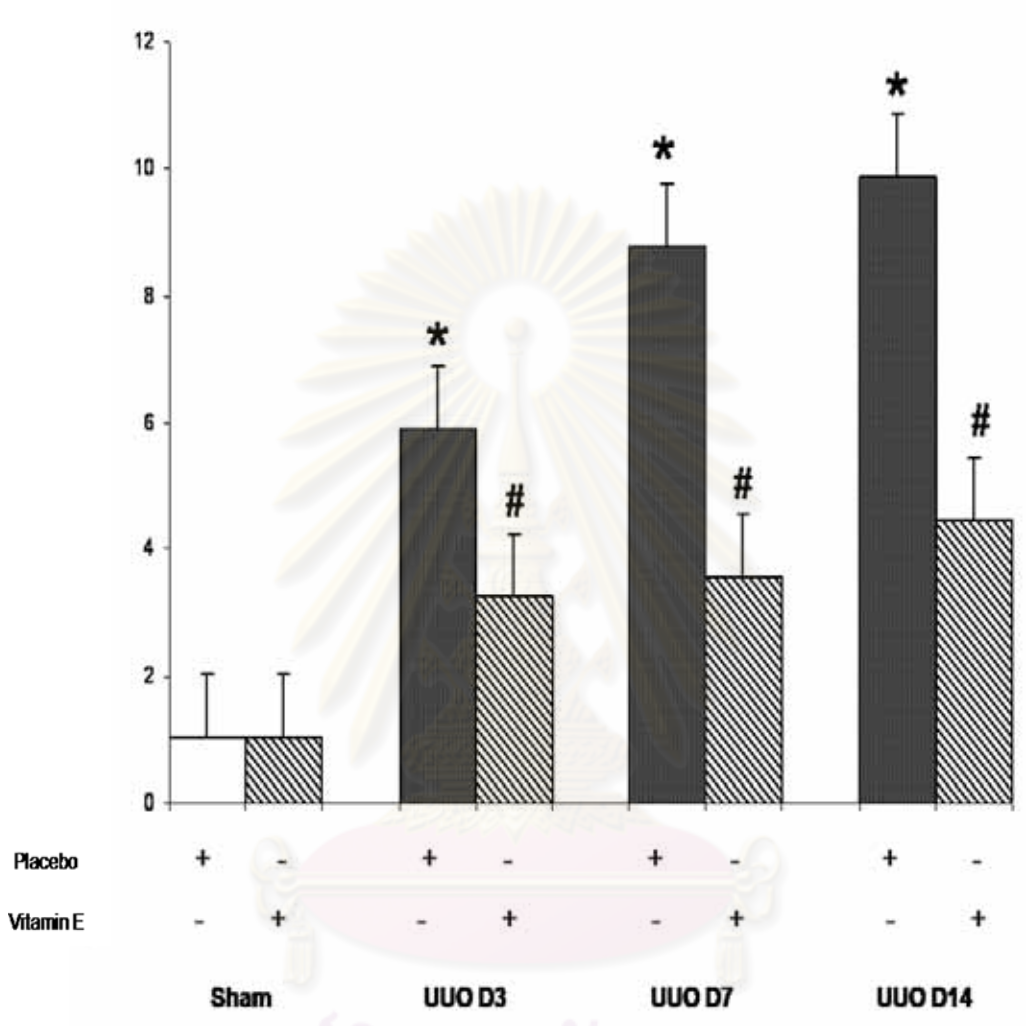


Figure 4.9 Real time RT-PCR for TGF-β1 mRNA expression in UUO mice. TGF-β1 mRNA expression showed markedly progressive upregulation in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E and significantly downregulation in the all vitamin E treated groups compared with placebo treatment groups. *$P < 0.05$ *vs* sham group; #$P < 0.05$ *vs* UUO with placebo treatment group.

4.4 Vitamin E treatment attenuated increased expression of Smad2/3

In order to investigate the effect of vitamin E responded to TGF-β/pro-fibrosis Smad2/3 during UUO. In sham kidneys, the author could not identify staining of Smad2/3 (Figure 4.10, A) in the nucleus of TEC, whereas the labeling of Smad2/3 was prominent in nucleus of TEC particularly in dilated and atrophic tubules of the placebo treated UUO kidneys since day 3 after UUO (Figure 4.10, B) and development until day 14 (Figure 4.10, C and D). In contrast, vitamin E treatment in mice with UUO demonstrated the significantly attenuated the nucleus staining intensity of Smad2/3 in the obstructed kidneys (Figure 4.10, E - H). Next, the author examined the effects of vitamin E treatment in UUO mice on protein and mRNA expression in TGF-β/pro-fibrosis Smad2/3 signaling pathway. Compatible with the immunohistochemistry, Western blot analysis demonstrated the significantly increased Smad2/3 protein levels in the obstructed kidneys with placebo treatment compared with sham kidneys ($P<0.05$) (Figure 4.11). In contrast, UUO mice with vitamin E treatment showed significantly inhibited the increasing of the Smad2/3 protein ($P<0.05$) (Figure 4.11) compared with the placebo treated UUO mice. Like a mirror, upregulation of Smad3 mRNA expression was significantly changed in any time course during obstructive process ($P<0.05$) (Figure 4.12). On the other hand, treatment with vitamin E in UUO mice showed significantly suppressed the upregulation of Smad3 mRNA expression, compared with placebo treated UUO mice ($P<0.05$) (Figure 4.12).

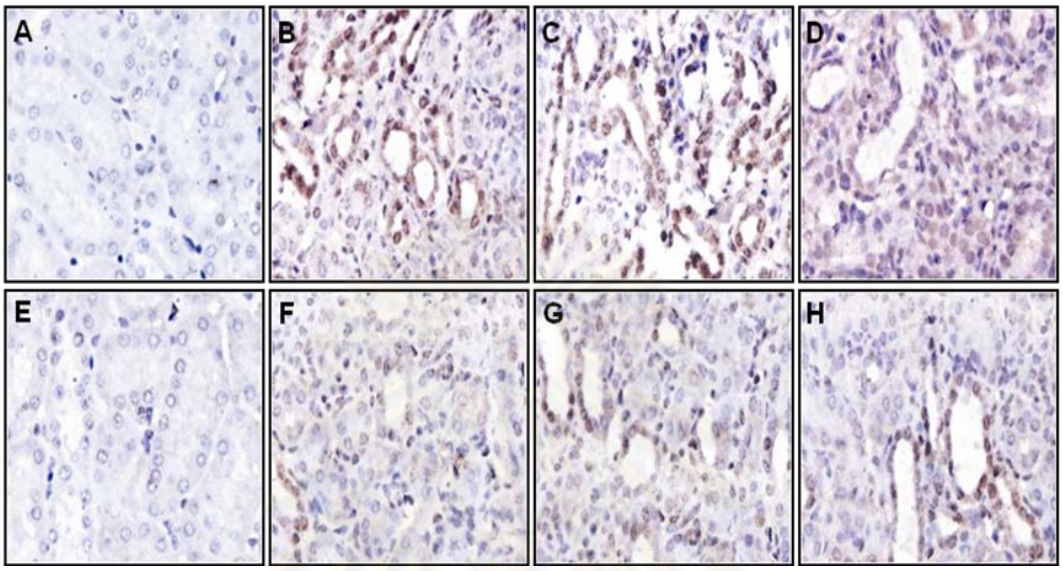


Figure 4.10 Immunohistochemical labeling of Smad2/3 in UUO mice. (A) In sham group, no Smad2/3 staining was detected in the kidneys. In obstructed kidney, advance increasing of Smad2/3 staining was seen in nuclei of TEC and some of interstitial inflammatory cells within the fibrosing obstructed kidneys compared with the sham control at day 3 (B), day 7 (C), and day 14 (D). However, decreasing of Smad2/3 expression was observed in UUO mice with vitamin E treatment at day 3 (F), day 7 (G), and day 14 (H) compared with placebo treatment groups.

Smad 2/3 protein in any time course

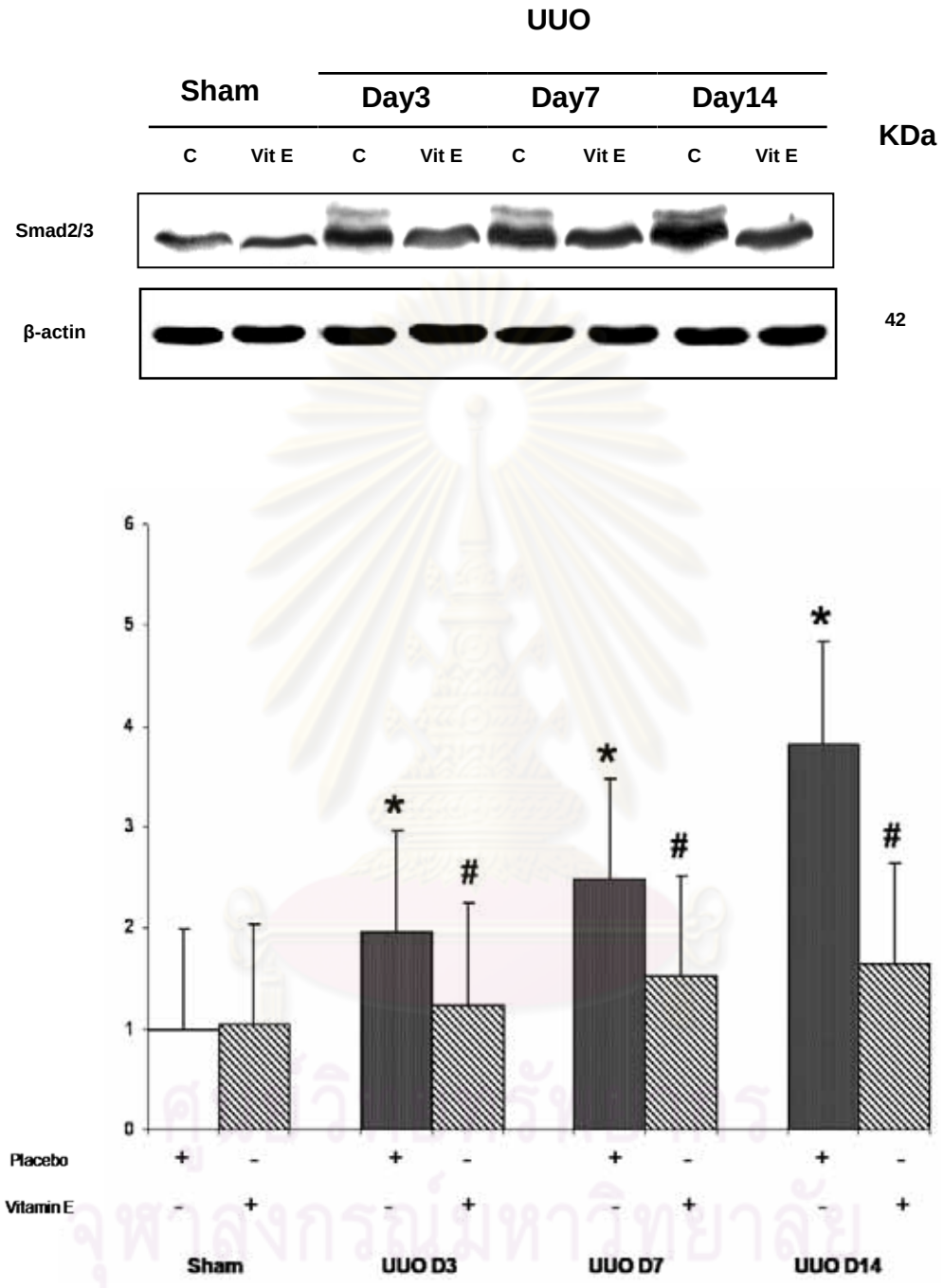


Figure 4.11 Western blot analysis for Smad2/3 protein expression in UUO mice. Smad2/3 protein expression was significantly increased in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. Treatment with vitamin E resulted in a decrease in the levels of Smad2/3. *$P < 0.05$ *vs* sham group; $^{\#}P < 0.05$ *vs* UUO with placebo treatment group.

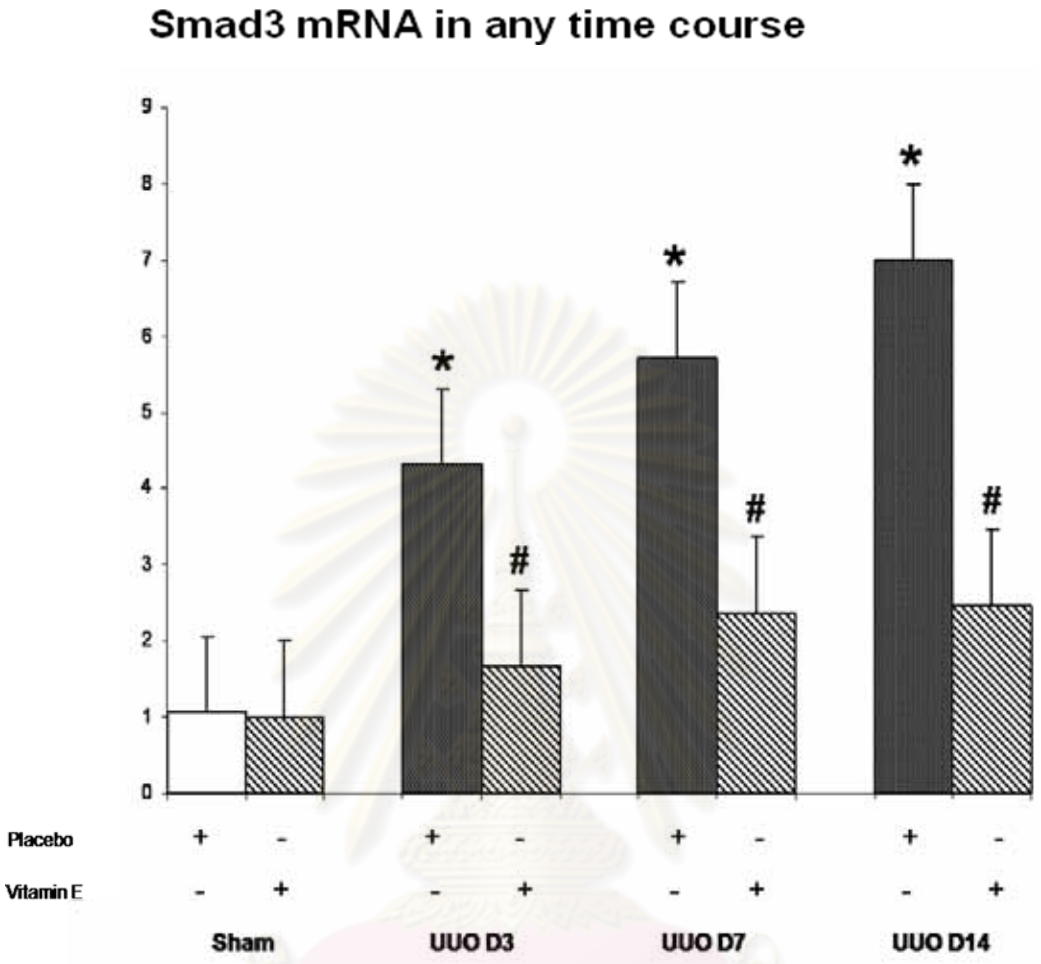


Figure 4.12 Real time RT-PCR for Smad3 mRNA expression in UUO mice. Smad3 mRNA expression showed markedly progressive upregulation in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E and significantly downregulation in the all vitamin E treated groups compared with placebo treatment group. *$P < 0.05$ *vs* sham group; #$P < 0.05$ *vs* UUO with placebo treatment group.

4.5 Vitamin E treatment delayed decreased expression of BMP-7 in UUO kidneys

In sham kidneys, the author demonstrated the staining of BMP-7 (Figure 4.13, A) in the cytoplasm of TEC, whereas the labeling of BMP-7 was decreased in cytoplasm of TEC particularly in dilated and atrophic tubules of the placebo treated UUO kidneys since day 3 after UUO (Figure 4.13, B) and progressively loss until day 14 (Figure 4.13, C and D). In contrast, vitamin E treatment in mice with UUO demonstrated the significantly attenuated the cytoplasm staining intensity of BMP-7 in the obstructed kidneys (Figure 4.13, E - H). In addition, the author examined the effects of vitamin E treatment in UUO mice on protein and mRNA expression in BMP-7. From Western blot analysis, the author demonstrated the significantly decreased BMP-7 protein levels in the obstructed kidneys with placebo treatment compared with sham kidneys ($P<0.05$) (Figure 4.14). In contrast, UUO mice with vitamin E treatment showed significantly delayed the declining of BMP-7 protein ($P<0.05$) (Figure 4.14) compared with the placebo treated UUO mice. Moreover, downregulation of BMP-7 mRNA expression was significantly changed in any time course during obstructive process ($P<0.05$) (Figure 4.15). In contrast, treatment with vitamin E in UUO mice showed significantly maintained the downregulation of BMP-7 mRNA expression, compared with placebo treatment UUO mice ($P<0.05$) (Figure 4.15).

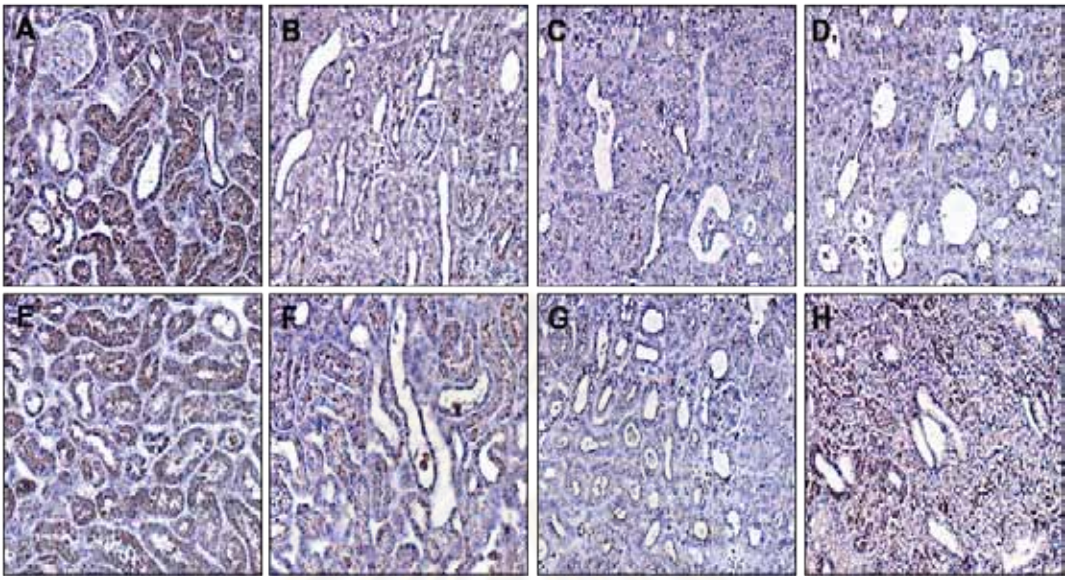


Figure 4.13 Immunohistochemical labeling of BMP-7 in UUO mice. (A) In sham-operated control kidneys, BMP-7 was detected in the cytoplasm of TEC, whereas the labeling of BMP-7 was decreased in cytoplasm of TEC particularly in dilated and atrophic tubules of the UUO kidneys compared with the sham at day 3 (B), day 7 (C), and day 14 (D). (E) In sham + vitamin E kidneys, BMP-7 staining was seen similar to the sham group. In contrast, staining of BMP-7 was stronger in UUO mice with vitamin E treatment at day 3 (F), day 7 (G), and day 14 (H) compared to placebo treatment groups.

BMP-7 protein in any time course

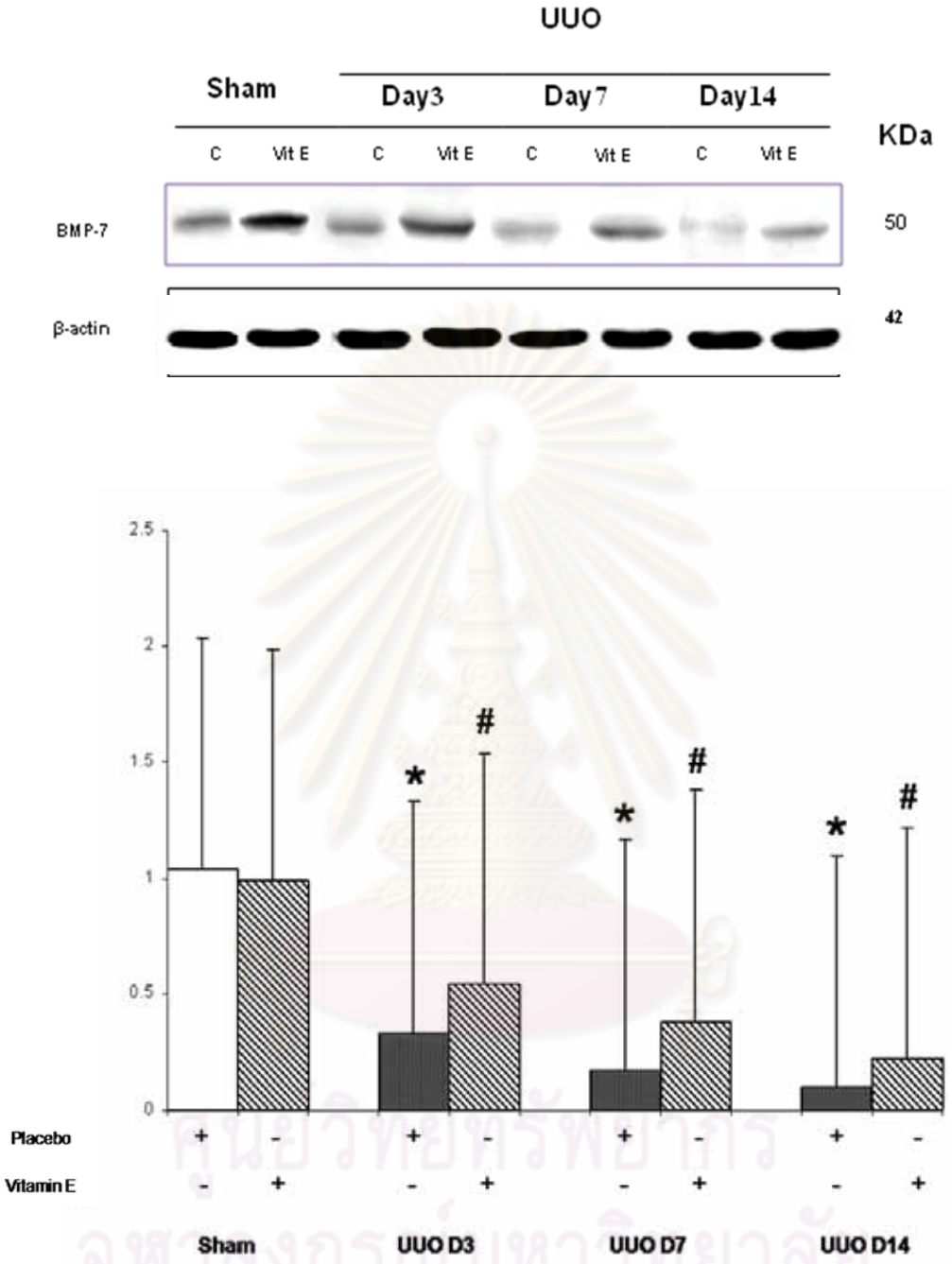


Figure 4.14 Western blot analysis for BMP-7 protein expression in UUO mice. Significantly progressive decreased BMP-7 protein expression was demonstrated in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. Treatment with vitamin E resulted in the maintain levels of BMP-7 protein compared with placebo treatment groups. *$P < 0.05$ *vs* sham group; #$P < 0.05$ *vs* UUO with placebo treatment group.

BMP-7 mRNA in any time course

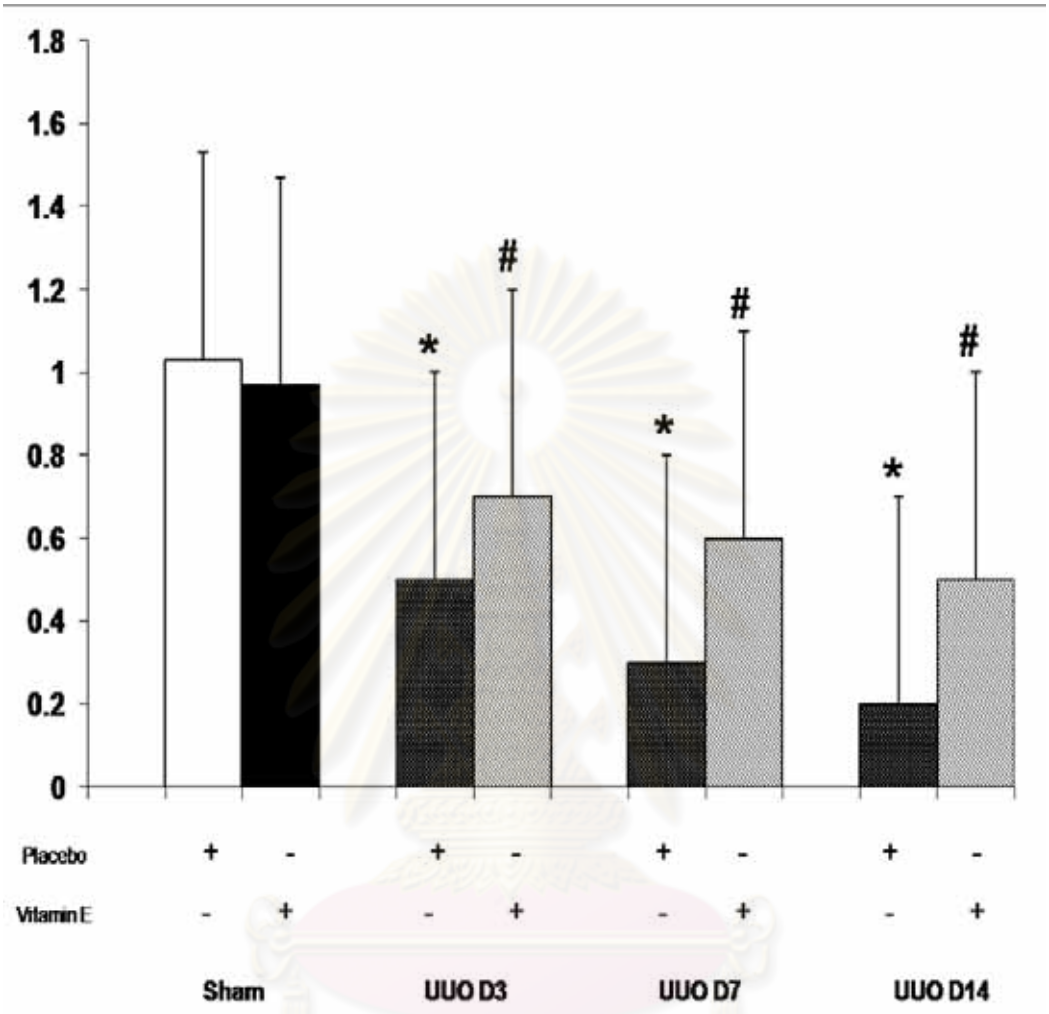


Figure 4.15 Real time RT-PCR for BMP-7 mRNA expression in UUO mice. BMP-7 mRNA showed significantly downregulation in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. In contrast, treatment with vitamin E in UUO mice showed significantly delays the downregulation of BMP-7 mRNA expression, compared with placebo treatment group. *$P < 0.05$ *vs* sham group; $^{\#}P < 0.05$ *vs* UUO with placebo treatment group.

4.6 Vitamin E treatment delayed the declining of Smad 1/5/8 expression in UUO kidneys

The author examined the effects of vitamin E treatment in UUO mice on protein and mRNA expression in BMP-7/anti-fibrosis Smad1/5/8 signaling pathway. From Western blot analysis, the author demonstrated the significantly decreased Smad1/5/8 protein levels in the obstructed kidneys with placebo treatment compared with sham kidneys ($P<0.05$) (Figure 4.16). In contrast, UUO mice with vitamin E treatment showed significantly delayed the declining of Smad1/5/8 protein, ($P<0.05$) (Figure 4.16) compared with the placebo treated UUO mice. Furthermore, downregulation of Smad8 mRNA expression was significantly changed in any time course during obstructive process ($P<0.05$) (Figure 4.17). On the other hand, treatment with vitamin E in UUO mice showed significantly maintains the downregulation of Smad8 mRNA expression, compared with placebo treatment UUO mice ($P<0.05$) (Figure 4.17).

Smad 1/5/8 protein in any time course

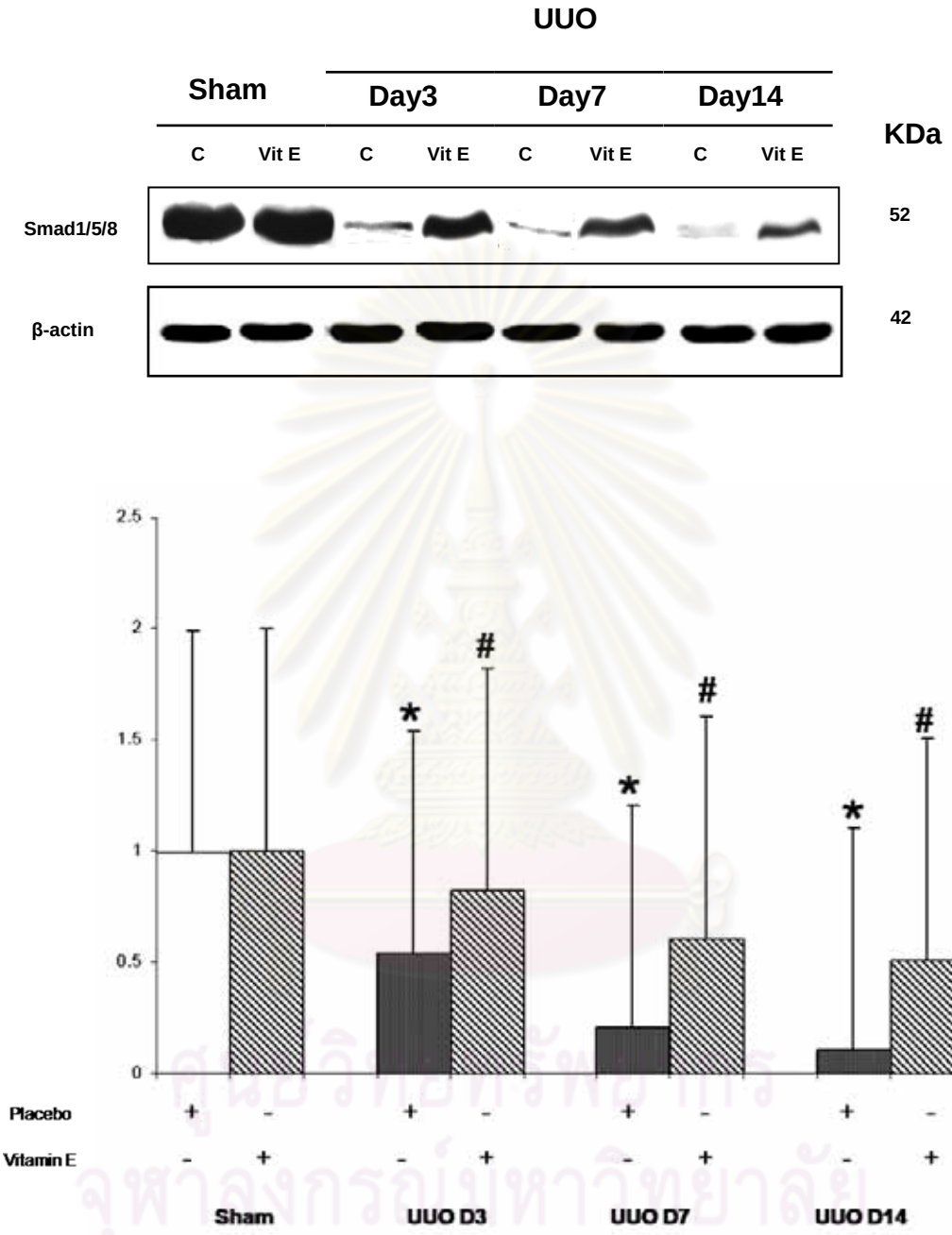


Figure 4.16 Western blot analysis for Smad1/5/8 protein expression in UUO mice. Significantly progressive decreased Smad1/5/8 protein expression was demonstrated in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. Treatment with vitamin E resulted in the maintain levels of Sman1/5/8 protein compared with placebo treatment groups. $^{*}P < 0.05$ *vs* sham group; $^{\#}P < 0.05$ *vs* UUO with placebo treatment group.

Smad8 mRNA in any time course

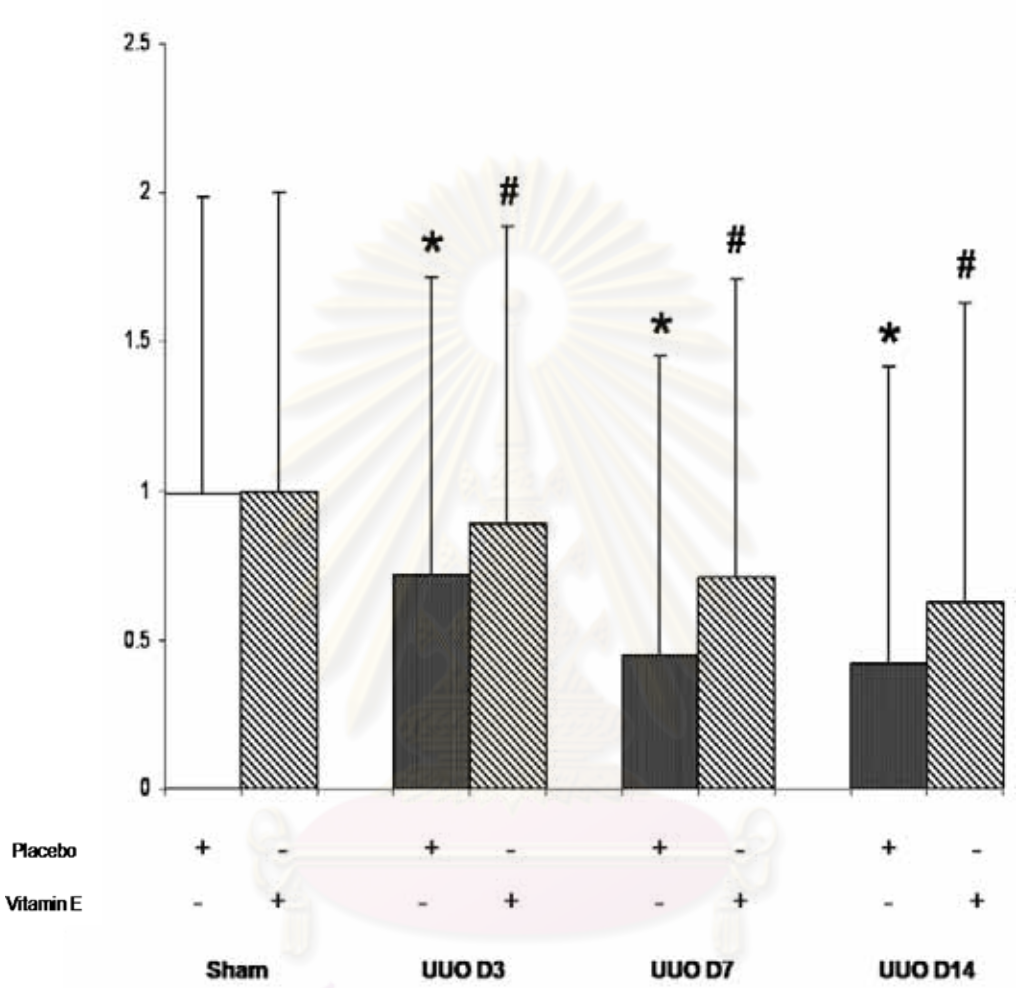


Figure 4.17 Real time RT-PCR for Smad8 mRNA expression in UUO mice. Smad8 mRNA showed significantly downregulation in UUO mice on day 3, day 7, and day 14 compared with sham or sham + vitamin E. On the other hand, treatment with vitamin E in UUO mice showed significantly sluggish the downregulation of Smad8 mRNA expression, compared with placebo treatment group. $^{*}P < 0.05$ *vs* sham group; $^{\#}P < 0.05$ *vs* UUO with placebo treatment group.

CHAPTER V

DISCUSSION

CKD is becoming a major public health problem worldwide. The current burden of disease might due to a change of the underlying pathogenicity of CKD. During chronic kidney injury, proliferation, apoptosis, and EMT of TECs beside tubulointerstitial infiltration of inflammatory cells are well known characteristic of UUO model (50, 104). Most striking feature observed after UUO is the development of TA/IF in the progression of renal diseases (54). Importantly, a large proportion of the interstitial fibroblasts are known to be originated from the TEC through the process of EMT in the progression to the renal fibrosis. Many studies in vivo and vitro demonstrated that TGF-β1 is the most powerful cytokine which induced EMT (74, 75). In contrast, BMP-7 has the opposite interaction and counteract with TGF-β1 to support TEC function and architecture (16, 83). Even though, any specific therapies that inhibit the progression of CKD are unavailable from any revisions. Promoting regeneration and inhibiting EMT of TEC should be the best therapeutic options which potentially retard renal fibrosis. The present study, the author provided evidence that anti-inflammatory capacity of vitamin E was able to suppress TGF-β1 expression unlike to preserve BMP-7 level in the obstructed kidneys. These results demonstrated that the reno-protective effect of vitamin E could slow the progression of TA/IF in UUO mice. Suppress EMT by vitamin E was demonstrated by deceasing of S100A4 that represent mesenchymal phenotypic change of TEC. Ameliorate fibrosis by blockade EMT could be mediated by attenuation of the TGF-β1 induced pro-fibrosis Smad2/3 but maintain BMP-7 induced anti-fibrosis Smad1/5/8 signaling pathway. Our findings suggested that treatment with vitamin E could be applied to inhibit the development of renal fibrosis by attenuating EMT and supporting regeneration of TEC from the imbalance of TGF-β1/BMP-7 induced Smad signaling pathway.

5.1 Upregulation of S100A4 expression in obstructed kidneys associated with TA/IF in UUO Mice

Striking pathology during chronic renal injury is TA/IF that related with loss of renal function. Interstitial fibroblasts can originate from TEC at the site of injury through EMT and are the principal source of kidney fibrosis (50). S100A4 is one of the important cytoskeleton markers of mesenchyme. In present study, immunohistochemistry staining revealed that expression of early mesenchymal cell marker using S100A4 was increased in the obstructed kidneys since establish the process of injury. Not only, this finding was confirmed with protein and gene analysis but also this process was on going through any time course during obstruction. The expression of S1004 considered specific for myofibroblasts are found in the interstitium area of obstructed kidney that may be derived from EMT and such cells may produce ECM deposition. S100A4 has been the characteristic of an early fibroblast marker in development of renal fibrosis and has been labeled in some TEC (66, 105) indicate cell motility and invasive capacity during EMT. These findings suggested that during the process of chronic kidney injury, TEC could be transdifferentiated into interstitial fibroblasts cause progression of renal fibrosis.

5.2 Upregulation of TGF-β1 and Smad2/3 expression in UUO kidneys associated with EMT

In present study, the author used the UUO model in mice to induce TA/IF that develop progression of renal fibrosis. The author demonstrated that UUO induced an increase of TGF-β1 protein and gene expression in the obstructed kidney as shown in the interstitial area by immunohistochemistry staining. Because TGF-β is known to be a major cytokine that regulate EMT, so the increasing of TGF-β1 in the obstructed kidneys could be involved in the loss of the epithelial phenotype and the achievement of the mesenchymal phenotype. TGF-β is a multifunctional cytokine that control various cellular processes, such as proliferation, apoptosis, growth arrest, and renal fibrosis through EMT (106). Many studies demonstrated that TGF-β promotes renal fibrosis through EMT by activation of pro-fibrosis Smad2/3 signals (79, 107, 108). In contrast, this process was counteracted with BMP-7 induced anti-fibrosis Smad1/5/8 (16, 83). In present study, the author provided the evidence that increasing of Smad2/3 protein and gene

expression could be transducer signal from TGF-β. The expressions of Smad2 and Smad3 protein have been shown major in nucleus and some cytoplasm of TEC. These mean that stimulation by TGF-β through transmembrane TGF-β receptor at TEC surface leading to phosphorylation and activation of endogenous Smad2 protein production mainly in cytoplasm of TEC and transduce the signal into TEC nucleus regulate in the part of Smad3 stimulation (109). So, upregulation of TGF-β induced pro-fibrosis Smad2/3 expression in UUO could be the major factor induce EMT. These data support the notion that TGF-β/Smad signaling is regulated by the many dynamic processes in TEC during EMT.

5.3 Downregulation of BMP-7 and Smad1/5/8 expression in UUO kidneys associated with EMT

During kidney development, BMP-7 play the important role to promote MET induce nephron formation. In addition, BMP-7 was demonstrated to maintain the epithelial phenotype of TEC (58). In present study, obstructed kidney turn to decrease BMP-7 protein in TEC by immunohistochemistry staining and was confirmed by western blot analysis. Moreover, BMP-7 gene expression was downregulation in any time course during UUO. These results could reduce the protective mechanism of TEC by losing BMP-7 and TEC change to be the mesenchymal cell consequently. Furthermore, the author demonstrated that Smad1/5/8 protein and Smad8 gene expression were decreased in fibrotic kidney during process of EMT similar to BMP-7 expression. These data could represent the protective effect of BMP-7 induced anti-fibrosis Smad1/5/8 during chronic kidney injury. Loss of these signals could promote the EMT.

5.4 Vitamin E treatment can attenuated TA/IF in obstructed kidneys and downregulation of S100A4 in UUO Mice

The present study investigated the reno-protective effect of vitamin E on renal injury leading to renal fibrosis in UUO model. Kidneys with ureteral obstruction developed progressive TA/IF damage led to increased ECM deposition in any time course of UUO. These pathological changes correlated with strongly induced expression of S100A4 marker of activated myofibroblast in the kidney. Administration of

vitamin E markedly reduced the deposition of ECM after UUO in the obstructed kidneys and inhibited S100A4 expression. Our results suggested that vitamin E can inhibit the development of renal fibrosis during chronic kidney injury.

5.5 Vitamin E treatment demonstrated renoprotective effects in UUO kidneys by inhibiting TGF-β and preserving BMP-7 to ameliorate EMT

Vitamin E has been proposed for the prevention or treatment of numerous health problems, which is primarily due to its antioxidant and anti-inflammatory properties (21). In present study, the vitamin E treatment effectively reduced TA/IF in UUO mice. Moreover, treatment with vitamin E can reduce the S100A4 protein and mRNA expression meaning slow the development of mesenchymal phenotype of TECs. In addition, treatment with vitamin E can suppress the upregulation of TGF-β1 during time course of UUO similar to many studies during acute and chronic inflammation (19, 22). These evidences confirmed the advantage of vitamin E treatment can inhibit the process of TGF-β induced EMT. In contrast, treatment with vitamin E can preserve BMP-7 leading to ameliorate EMT. Furthermore, the present study provided the evidence that vitamin E can suppress pro-fibrosis of TGF-β1 induced Smad2/3 and maintain anti-fibrosis of BMP-7 induced Smad 1/5/8 signals in mice UUO also. These effects could be the benefit of vitamin E treatment to protect the kidney from chronic injury by modifies the notion of TGF-β and BMP-7/Smad signaling like a concert that regulated TEC by many dynamic processes during EMT.

CHAPTER VI

CONCLUSION

In conclusion, EMT is the most important mechanism that induces the progression of renal fibrosis. During chronic inflammation in the kidney, stimulation of pro-fibrotic TGF-β1/Smad2/3 but inhibition of anti-fibrotic BMP-7/Smad1/5/8 signaling pathway caused the development of fibrotic kidneys. Interestingly, anti-inflammation property of vitamin E could ameliorate renal fibrosis by inhibiting pro-fibrotic TGF-β1/Smad2/3 and preserving anti-fibrotic BMP-7/Smad1/5/8 signals in the obstructed kidneys. Thus, vitamin E could be the alternative therapeutic approach in the future to the clinical application in CKD patients.

REFERENCES

[1] Obrador GT, Garcia-Garcia G, Villa AR, Rubilar X, Olvera N, Ferreira E, et al. Prevalence of chronic kidney disease in the Kidney Early Evaluation Program (KEEP) Mexico and comparison with KEEP US. **Kidney Int Suppl.** Mar(116):S2-8.

[2] Donovan K, Ford D, van Schalkwyk D, Ansell D. UK Renal Registry 12th Annual Report (December 2009): chapter 16: international comparisons with the UK RRT programme. **Nephron Clin Pract.**115 Suppl 1:c309-19.

[3] Shaheen FA, Souqiyyeh MZ. Kidney health in the Middle East. **Clin Nephrol.** Nov;74 Suppl 1:S85-8.

[4] Cosio FG, Alamir A, Yim S, Pesavento TE, Falkenhain ME, Henry ML, et al. Patient survival after renal transplantation: I. The impact of dialysis pre-transplant. **Kidney Int.** 1998 Mar;53(3):767-72.

[5] Kanda E, Erickson K, Bond TC, Krisher J, McClellan WM. Hemodialysis Treatment Center Early Mortality Rates for Incident Hemodialysis Patients Are Associated with the Quality of Care prior to Starting but Not following Onset of Dialysis. **Am J Nephrol.** 2011 Apr 5;33(5):390-7.

[6] Vonesh EF, Snyder JJ, Foley RN, Collins AJ. Mortality studies comparing peritoneal dialysis and hemodialysis: what do they tell us? **Kidney Int Suppl.** 2006 Nov(103):S3-11.

[7] Barsoum RS. Chronic kidney disease in the developing world. **N Engl J Med.** 2006 Mar 9;354(10):997-9.

[8] Stevens LA, Viswanathan G, Weiner DE. Chronic kidney disease and end-stage renal disease in the elderly population: current prevalence, future projections, and clinical significance. **Adv Chronic Kidney Dis.** 2010 Jul;17(4):293-301.

[9] Sica DA. Pharmacologic Issues in treating hypertension in CKD. **Adv Chronic Kidney Dis.** 2011 Jan;18(1):42-7.

[10] Woo KT, Wong KS, Chan CM. Clinical trials of the past decade in the management of chronic kidney disease. **Rev Recent Clin Trials.** 2009 Sep;4(3):159-62.

[11] Iwano M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. **Curr Opin Nephrol Hypertens.** 2004 May;13(3):279-84.

[12] Rodriguez-Iturbe B, Johnson RJ, Herrera-Acosta J. Tubulointerstitial damage and progression of renal failure. **Kidney Int Suppl.** 2005 Dec(99):S82-6.

[13] Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. **J Am Soc Nephrol.** 2004 Jan;15(1):1-12.

[14] Liu Y. New insights into epithelial-mesenchymal transition in kidney fibrosis. **J Am Soc Nephrol.** 2010 Feb;21(2):212-22.

[15] Neilson EG. Setting a trap for tissue fibrosis. **Nat Med.** 2005 Apr;11(4):373-4.

[16] Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, et al. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. **Nat Med.** 2003 Jul;9(7):964-8.

[17] Wang S, Hirschberg R. Bone morphogenetic protein-7 signals opposing transforming growth factor beta in mesangial cells. **J Biol Chem.** 2004 May 28;279(22):23200-6.

[18] Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. **Annu Rev Nutr.** 1990;10:357-82.

[19] Jenkins JK, Huang H, Ndebele K, Salahudeen AK. Vitamin E inhibits renal mRNA expression of COX II, HO I, TGFbeta, and osteopontin in the rat model of cyclosporine nephrotoxicity. **Transplantation.** 2001 Jan 27;71(2):331-4.

[20] Lee IM, Cook NR, Gaziano JM, Gordon D, Ridker PM, Manson JE, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. **JAMA.** 2005 Jul 6;294(1):56-65.

[21] Singh U, Devaraj S, Jialal I. Vitamin E, oxidative stress, and inflammation. **Annu Rev Nutr.** 2005;25:151-74.

[22] Wang QL, Yuan JL, Tao YY, Zhang Y, Liu P, Liu CH. Fuzheng Huayu recipe and vitamin E reverse renal interstitial fibrosis through counteracting TGF-beta1-induced epithelial-to-mesenchymal transition. **J Ethnopharmacol.** Feb 17;127(3):631-40.

[23] Levin A. Clinical epidemiology of cardiovascular disease in chronic kidney disease prior to dialysis. **Semin Dial.** 2003 Mar-Apr;16(2):101-5.

[24] Stenvinkel P. Chronic kidney disease: a public health priority and harbinger of premature cardiovascular disease. **J Intern Med.** Nov;268(5):456-67.

[25] Black C, Sharma P, Scotland G, McCullough K, McGurn D, Robertson L, et al. Early referral strategies for management of people with markers of renal disease: a systematic review of the evidence of clinical effectiveness, cost-effectiveness and economic analysis. **Health Technol Assess.** Apr;14(21):1-184.

[26] Pereira BJ. Overcoming barriers to the early detection and treatment of chronic kidney disease and improving outcomes for end-stage renal disease. **Am J Manag Care.** 2002 Mar;8(4 Suppl):S122-35; quiz S36-9.

[27] Srisawat N, Manotham K, Eiam-Ong S, Katavetin P, Praditpornsilpa K. Erythropoietin and its non-erythropoietic derivative: do they ameliorate renal tubulointerstitial injury in ureteral obstruction? **Int J Urol.** 2008 Oct;15(11):1011-7.

[28] Vieira JM, Jr., Mantovani E, Rodrigues LT, Delle H, Noronha IL, Fujihara CK, et al. Simvastatin attenuates renal inflammation, tubular transdifferentiation and interstitial fibrosis in rats with unilateral ureteral obstruction. **Nephrol Dial Transplant.** 2005 Aug;20(8):1582-91.

[29] Cases A, Coll E. Dyslipidemia and the progression of renal disease in chronic renal failure patients. **Kidney Int Suppl.** 2005 Dec(99):S87-93.

[30] Bahlmann FH, Kielstein JT, Haller H, Fliser D. Erythropoietin and progression of CKD. **Kidney Int Suppl.** 2007 Nov(107):S21-5.

[31] K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease. **Am J Kidney Dis.** 2003 Oct;42(4 Suppl 3):S1-201.

[32] Levey AS, Eckardt KU, Tsukamoto Y, Levin A, Coresh J, Rossert J, et al. Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). **Kidney Int.** 2005 Jun;67(6):2089-100.

[33] Perneger TV, Brancati FL, Whelton PK, Klag MJ. End-stage renal disease attributable to diabetes mellitus. **Ann Intern Med.** 1994 Dec 15;121(12):912-8.

[34] Haroun MK, Jaar BG, Hoffman SC, Comstock GW, Klag MJ, Coresh J. Risk factors for chronic kidney disease: a prospective study of 23,534 men and women in Washington County, Maryland. **J Am Soc Nephrol.** 2003 Nov;14(11):2934-41.

[35] Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, et al. Prevalence of chronic kidney disease in the United States. **JAMA.** 2007 Nov 7;298(17):2038-47.

[36] Snyder JJ, Foley RN, Collins AJ. Prevalence of CKD in the United States: a sensitivity analysis using the National Health and Nutrition Examination Survey (NHANES) 1999-2004. **Am J Kidney Dis.** 2009 Feb;53(2):218-28.

[37] Coresh J, Astor BC, Greene T, Eknoyan G, Levey AS. Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. **Am J Kidney Dis.** 2003 Jan;41(1):1-12.

[38] Coresh J, Byrd-Holt D, Astor BC, Briggs JP, Eggers PW, Lacher DA, et al. Chronic kidney disease awareness, prevalence, and trends among U.S. adults, 1999 to 2000. **J Am Soc Nephrol.** 2005 Jan;16(1):180-8.

[39] Chittinandana A, Chailimpamontree W, Chaloeiphap P. Prevalence of chronic kidney disease in Thai adult population. **J Med Assoc Thai.** 2006 Aug;89 Suppl 2:S112-20.

[40] Perkovic V, Cass A, Patel AA, Suriyawongpaisal P, Barzi F, Chadban S, et al. High prevalence of chronic kidney disease in Thailand. **Kidney Int.** 2008 Feb;73(4):473-9.

[41] Ingsathit A, Thakkinstian A, Chaiprasert A, Sangthawan P, Gojaseni P, Kiattisunthorn K, et al. Prevalence and risk factors of chronic kidney disease in the Thai adult population: Thai SEEK study. **Nephrol Dial Transplant.** May;25(5):1567-75.

[42] Jono S, Shioi A, Ikari Y, Nishizawa Y. Vascular calcification in chronic kidney disease. **J Bone Miner Metab.** 2006;24(2):176-81.

[43] Bleyer AJ, Sedor JR, Freedman BI, O'Brien A, Russell GB, Graley J, et al. Risk factors for development and progression of diabetic kidney disease and

treatment patterns among diabetic siblings of patients with diabetic kidney disease. **Am J Kidney Dis.** 2008 Jan;51(1):29-37.

[44] Torffvit O, Agardh CD. The impact of metabolic and blood pressure control on incidence and progression of nephropathy. A 10-year study of 385 type 2 diabetic patients. **J Diabetes Complications.** 2001 Nov-Dec;15(6):307-13.

[45] Schaeffner ES, Kurth T, Bowman TS, Gelber RP, Gaziano JM. Blood pressure measures and risk of chronic kidney disease in men. **Nephrol Dial Transplant.** 2008 Apr;23(4):1246-51.

[46] Zeisberg M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. **J Am Soc Nephrol.** 2010 Nov;21(11):1819-34.

[47] Rodriguez-Iturbe B, Garcia Garcia G. The role of tubulointerstitial inflammation in the progression of chronic renal failure. **Nephron Clin Pract.** 2010;116(2):c81-8.

[48] Sharma K, Cook A, Smith M, Valancius C, Inscho EW. TGF-beta impairs renal autoregulation via generation of ROS. **Am J Physiol Renal Physiol.** 2005 May;288(5):F1069-77.

[49] Fine LG, Norman JT. Chronic hypoxia as a mechanism of progression of chronic kidney diseases: from hypothesis to novel therapeutics. **Kidney Int.** 2008 Oct;74(7):867-72.

[50] Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. **J Clin Invest.** 2002 Aug;110(3):341-50.

[51] Iseki K, Ikemiya Y, Iseki C, Takishita S. Proteinuria and the risk of developing end-stage renal disease. **Kidney Int.** 2003 Apr;63(4):1468-74.

[52] Morigi M, Macconi D, Zoja C, Donadelli R, Buelli S, Zanchi C, et al. Protein overload-induced NF-kappaB activation in proximal tubular cells requires

H(2)O(2) through a PKC-dependent pathway. **J Am Soc Nephrol.** 2002 May;13(5):1179-89.

[53] Li Y, Spataro BC, Yang J, Dai C, Liu Y. 1,25-dihydroxyvitamin D inhibits renal interstitial myofibroblast activation by inducing hepatocyte growth factor expression. **Kidney Int.** 2005 Oct;68(4):1500-10.

[54] Klahr S, Morrissey J. Obstructive nephropathy and renal fibrosis. **Am J Physiol Renal Physiol.** 2002 Nov;283(5):F861-75.

[55] Schmidt-Ott KM, Lan D, Hirsh BJ, Barasch J. Dissecting stages of mesenchymal-to-epithelial conversion during kidney development. **Nephron Physiol.** 2006;104(1):p56-60.

[56] Karsenty G, Luo G, Hofmann C, Bradley A. BMP 7 is required for nephrogenesis, eye development, and skeletal patterning. **Ann N Y Acad Sci.** 1996 Jun 8;785:98-107.

[57] Godin RE, Robertson EJ, Dudley AT. Role of BMP family members during kidney development. **Int J Dev Biol.** 1999;43(5):405-11.

[58] Patel SR, Dressler GR. BMP7 signaling in renal development and disease. **Trends Mol Med.** 2005 Nov;11(11):512-8.

[59] Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. **J Clin Invest.** 2003 Dec;112(12):1776-84.

[60] Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. **Nat Rev Mol Cell Biol.** 2006 Feb;7(2):131-42.

[61] Kalluri R, Zeisberg M. Fibroblasts in cancer. **Nat Rev Cancer.** 2006 May;6(5):392-401.

[62] Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. **J Clin Invest.** 2009 Jun;119(6):1429-37.

[63] Hay ED, Zuk A. Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. **Am J Kidney Dis.** 1995 Oct;26(4):678-90.

[64] Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. **Curr Opin Cell Biol.** 2005 Oct;17(5):548-58.

[65] Strutz F, Zeisberg M, Ziyadeh FN, Yang CQ, Kalluri R, Muller GA, et al. Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation. **Kidney Int.** 2002 May;61(5):1714-28.

[66] Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, et al. Identification and characterization of a fibroblast marker: FSP1. **J Cell Biol.** 1995 Jul;130(2):393-405.

[67] Gabbiani G, Kapanci Y, Barazzone P, Franke WW. Immunochemical identification of intermediate-sized filaments in human neoplastic cells. A diagnostic aid for the surgical pathologist. **Am J Pathol.** 1981 Sep;104(3):206-16.

[68] Hynes RO, Yamada KM. Fibronectins: multifunctional modular glycoproteins. **J Cell Biol.** 1982 Nov;95(2 Pt 1):369-77.

[69] Essawy M, Soylemezoglu O, Muchaneta-Kubara EC, Shortland J, Brown CB, el Nahas AM. Myofibroblasts and the progression of diabetic nephropathy. **Nephrol Dial Transplant.** 1997 Jan;12(1):43-50.

[70] Eddy AA. Molecular basis of renal fibrosis. **Pediatr Nephrol.** 2000 Dec;15(3-4):290-301.

[71] Strutz FM. EMT and proteinuria as progression factors. **Kidney Int.** 2009 Mar;75(5):475-81.

[72] Yang J, Liu Y. Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. **Am J Pathol.** 2001 Oct;159(4):1465-75.

[73] Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. **J Cell Biol.** 1994 Dec;127(6 Pt 2):2021-36.

[74] Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. **Oncogene.** 2005 Aug 29;24(37):5764-74.

[75] Willis BC, Borok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. **Am J Physiol Lung Cell Mol Physiol.** 2007 Sep;293(3):L525-34.

[76] Li Y, Kang YS, Dai C, Kiss LP, Wen X, Liu Y. Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. **Am J Pathol.** 2008 Feb;172(2):299-308.

[77] Massague J, Gomis RR. The logic of TGFbeta signaling. **FEBS Lett.** 2006 May 22;580(12):2811-20.

[78] Feng XH, Derynck R. Specificity and versatility in tgf-beta signaling through Smads. **Annu Rev Cell Dev Biol.** 2005;21:659-93.

[79] Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. **J Clin Invest.** 2003 Nov;112(10):1486-94.

[80] Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP. Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. **EMBO J.** 2004 Mar 10;23(5):1155-65.

[81] Yang J, Dai C, Liu Y. A novel mechanism by which hepatocyte growth factor blocks tubular epithelial to mesenchymal transition. **J Am Soc Nephrol.** 2005 Jan;16(1):68-78.

[82] Dudley AT, Godin RE, Robertson EJ. Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme. **Genes Dev.** 1999 Jun 15;13(12):1601-13.

[83] Tyler JR, Robertson H, Booth TA, Burt AD, Kirby JA. Chronic allograft nephropathy: intraepithelial signals generated by transforming growth factor-beta and bone morphogenetic protein-7. **Am J Transplant.** 2006 Jun;6(6):1367-76.

[84] Reiter E, Jiang Q, Christen S. Anti-inflammatory properties of alpha- and gamma-tocopherol. **Mol Aspects Med.** 2007 Oct-Dec;28(5-6):668-91.

[85] Tucker JM, Townsend DM. Alpha-tocopherol: roles in prevention and therapy of human disease. **Biomed Pharmacother.** 2005 Aug;59(7):380-7.

[86] Singh U, Jialal I. Anti-inflammatory effects of alpha-tocopherol. **Ann N Y Acad Sci.** 2004 Dec;1031:195-203.

[87] Burton GW, Foster DO, Perly B, Slater TF, Smith IC, Ingold KU. Biological antioxidants. **Philos Trans R Soc Lond B Biol Sci.** 1985 Dec 17;311(1152):565-78.

[88] Zandi PP, Anthony JC, Khachaturian AS, Stone SV, Gustafson D, Tschanz JT, et al. Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study. **Arch Neurol.** 2004 Jan;61(1):82-8.

[89] Ricciarelli R, Maroni P, Ozer N, Zingg JM, Azzi A. Age-dependent increase of collagenase expression can be reduced by alpha-tocopherol via protein kinase C inhibition. **Free Radic Biol Med.** 1999 Oct;27(7-8):729-37.

[90] Teupser D, Thiery J, Seidel D. Alpha-tocopherol down-regulates scavenger receptor activity in macrophages. **Atherosclerosis**. 1999 May;144(1):109-15.

[91] Ricciarelli R, Zingg JM, Azzi A. Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells. **Circulation**. 2000 Jul 4;102(1):82-7.

[92] Islam KN, Devaraj S, Jialal I. alpha-Tocopherol enrichment of monocytes decreases agonist-induced adhesion to human endothelial cells. **Circulation**. 1998 Nov 24;98(21):2255-61.

[93] Martin A, Foxall T, Blumberg JB, Meydani M. Vitamin E inhibits low-density lipoprotein-induced adhesion of monocytes to human aortic endothelial cells in vitro. **Arterioscler Thromb Vasc Biol**. 1997 Mar;17(3):429-36.

[94] Yoshikawa T, Yoshida N, Manabe H, Terasawa Y, Takemura T, Kondo M. alpha-Tocopherol protects against expression of adhesion molecules on neutrophils and endothelial cells. **Biofactors**. 1998;7(1-2):15-9.

[95] Freedman JE, Farhat JH, Loscalzo J, Keaney JF, Jr. alpha-tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism. **Circulation**. 1996 Nov 15;94(10):2434-40.

[96] Saldeen T, Li D, Mehta JL. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. **J Am Coll Cardiol**. 1999 Oct;34(4):1208-15.

[97] Azzi A, Gysin R, Kempna P, Munteanu A, Negis Y, Villacorta L, et al. Vitamin E mediates cell signaling and regulation of gene expression. **Ann N Y Acad Sci**. 2004 Dec;1031:86-95.

[98] Zingg JM, Azzi A. Non-antioxidant activities of vitamin E. **Curr Med Chem**. 2004 May;11(9):1113-33.

[99] Card JW, Racz WJ, Brien JF, Massey TE. Attenuation of amiodarone-induced pulmonary fibrosis by vitamin E is associated with suppression of transforming growth factor-beta1 gene expression but not prevention of mitochondrial dysfunction. **J Pharmacol Exp Ther.** 2003 Jan;304(1):277-83.

[100] Liu H, Xiong M, Xia YF, Cui NJ, Lu RB, Deng L, et al. Studies on pentoxifylline and tocopherol combination for radiation-induced heart disease in rats. **Int J Radiat Oncol Biol Phys.** 2009 Apr 1;73(5):1552-9.

[101] Gomez JA, Molero X, Vaquero E, Alonso A, Salas A, Malagelada JR. Vitamin E attenuates biochemical and morphological features associated with development of chronic pancreatitis. **Am J Physiol Gastrointest Liver Physiol.** 2004 Jul;287(1):G162-9.

[102] Devaraj S, Jialal I. Low-density lipoprotein postsecretory modification, monocyte function, and circulating adhesion molecules in type 2 diabetic patients with and without macrovascular complications: the effect of alpha-tocopherol supplementation. **Circulation.** 2000 Jul 11;102(2):191-6.

[103] Devaraj S, Jialal I. Alpha tocopherol supplementation decreases serum C-reactive protein and monocyte interleukin-6 levels in normal volunteers and type 2 diabetic patients. **Free Radic Biol Med.** 2000 Oct 15;29(8):790-2.

[104] Truong LD, Petrusevska G, Yang G, Gurpinar T, Shappell S, Lechago J, et al. Cell apoptosis and proliferation in experimental chronic obstructive uropathy. **Kidney Int.** 1996 Jul;50(1):200-7.

[105] Guarino M, Tosoni A, Nebuloni M. Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. **Hum Pathol.** 2009 Oct;40(10):1365-76.

[106] Heldin CH, Landstrom M, Moustakas A. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. **Curr Opin Cell Biol.** 2009 Apr;21(2):166-76.

[107] Zhang D, Sun L, Xian W, Liu F, Ling G, Xiao L, et al. Low-dose paclitaxel ameliorates renal fibrosis in rat UUO model by inhibition of TGF-beta/Smad activity. **Lab Invest.** Mar;90(3):436-47.

[108] Inazaki K, Kanamaru Y, Kojima Y, Sueyoshi N, Okumura K, Kaneko K, et al. Smad3 deficiency attenuates renal fibrosis, inflammation,and apoptosis after unilateral ureteral obstruction. **Kidney Int.** 2004 Aug;66(2):597-604.

[109] Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. **Nature.** 1997 Dec 4;390(6659):465-71.

APPENDIX

STANDARD BUFFERS AND REAGENTS

Tris-buffered saline (1xTBS)

Tris Base	24.7 mM
NaCl	137 mM
KCl	2.7 mM

pH was adjusted to 7.4 at room temperature with HCl

Phosphate-buffered saline (1xPBS)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM

BCA microtitre protein assay reagents

Solution A	2% BCA
Solution B	2% Na_2CO_3, 0.16% sodium tartrate, 0.4% NaOH, 0.95% $NaHCO_3$, pH 11.25 (adjust pH with NaOH or solid $NaHCO_3$).
Solution C	4% $CuSO_4.5H_2O$

Standard denaturing Laemmli PAGE buffers

Resolving gel buffer	1.5 M Tris-Cl, pH 8.8, 0.4% SDS
Stacking gel buffer	1 M Tris-Cl, pH 6.8, 0.4% SDS

30% Acrylamide/Bis-acrylamide mix (37.5:1)

10% Ammonium persulphate (AMPS)

4xsample loading buffer	250 mM Tris-Cl, pH 6.8, 20% Glycerol, 4% SDS, 2 mM EDTA, 400 mM DTT, 4% β-mercaptoethanol and 0.01% bromophenol blue
10xElectrophoresis buffer	250 mM Tris, 1.92 M Glycine, 0.1% SDS

Blotting buffers

1xTowbin's transfer buffer

Tris Base	24 mM
Glycine	192 mM
Methanol	20%

1xBolt and Mahoney's transfer buffer

Tris base	40 mM
Na $O_2CH_3C_3H_2O$	20 mM
EDTA, free acid	2 mM
SDS	0.05% w/v
Methanol	20%

TBS/Tween

1xTBS containing 0.1% Tween 20

Stripping buffer

Tris pH 7.0	10 mM
SDS	1%
β-mercaptoethanol	0.01% v/v

Protein staining

Ponceau S stain

Ponceau-S	0.1%
Acetic acid	5%

Colloidal Coomassie G250 stain

Orthophosphoric acid	2.5%
Ammonium sulphate	8%
Coomassie G250	0.01%
Absolute ethanol	20%

30 ml Orthophosphoric acid was added to 750 ml distilled water with stirring, and then 80 g ammonium sulphate was added with continual stirring to dissolve all components. This solution was added with 2 ml 5% G250 and added volume into 800 ml with distilled water, finally 200 ml absolute ethanol (or methanol) was added.

Lysis buffers

Tissue cell line lysis buffer

Tris-HCl, pH 7.4	50 mM
NaCl	150 mM
Triton-X	0.5%
EDTA	1 mM

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