ผลของการยับยั้งระบบเรนิน-แองจิโอเทนซิน ต่อการทำหน้าที่ของไต ปริมาณนอร์อิพิเนฟรินในไต และความเครียดออกซิเดชั่นในไต ในหนูที่ถูกเหนี่ยวนำให้เกิดความเสียหายของไตด้วยไซโคลสปอริน

นางสาว สุวพร พนัสเจริญ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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EFFECTS OF RENIN-ANGIOTENSIN BLOCKADE ON RENAL FUNCTION, RENAL NOREPINEPHRINE CONTENTS AND OXIDATIVE STRESS IN CYCLOSPORINE INDUCED NEPHROSIS RATS

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สถาบันวิทยบริการ
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A Thesis Submitted in Partial Fulfillment of the Requirements
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สุวพร พนัสเจริญ : ผลของการยับยั้งระบบเรนิน-แองจิโอเทนซิน ต่อการทำหน้าที่ของไต ปริมาณนอร์อิพิเนฟรินในไต และความเครียดออกซิเดชั่นในไต ในหนูที่ถูกเหนี่ยวนำให้เกิดความเสียหายของไตด้วยไซโคลสปอริน. (EFFECTS OF RENIN-ANGIOTENSIN BLOCKADE ON RENAL FUNCTION, RENAL NOREPINEPHRINE CONTENTS AND OXIDATIVE STRESS IN CYCLOSPORINE INDUCED NEPHROSIS RATS.) อ. ที่ปรึกษา : รศ.สพ.ญ.ดร. ชลลดา บูรณกาล, อ. ที่ปรึกษาร่วม: ผศ.สพ.ญ.ดร. สฤณี กลันทกานนท์ ทองทรง, 62 หน้า.

วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อศึกษาผลของการยับยั้งระบบเรนิน-แองจิโอเทนซิน ต่อการทำหน้าที่ของไต ปริมาณนอร์อิพิเนฟรินในไต และความเครียดออกซิเดชั่นในไต ในหนูที่ถูกเหนี่ยวนำให้เกิดความเสียหายของไตด้วยไซโคลสปอริน โดยทำการทดลองในหนูซึ่งถูกแบ่งออกเป็น 3 กลุ่ม ดังนี้ กลุ่มที่ 1 (กลุ่มควบคุม) ได้รับโพไพรีนไกลคอลขนาด 1 มล. ต่อกก.น้ำหนักตัว ต่อวัน ฉีดเข้าใต้ผิวหนัง นาน 28 วัน กลุ่มที่ 2 (กลุ่มไซโคลสปอริน) ได้รับไซโคลสปอรินขนาด 15 มก. ต่อกก. น้ำหนักตัว ต่อวัน ฉีดเข้าใต้ผิวหนัง นาน 28 วัน และ กลุ่มที่ 3 (กลุ่มไซโคลสปอรินและโลซาทาน) ได้รับไซโคลสปอรินขนาด 15 มก. ต่อกก.น้ำหนักตัว ต่อวันฉีดเข้าใต้ผิวหนัง และโลซาทานขนาด 10 มก.ต่อกก.น้ำหนักตัว ต่อวันโดยการกิน นาน 28 วัน หลังจากนั้นจะนำมาหาการทำหน้าที่ของไต ปริมาณนอร์อิพิเนฟรินในไต และความเครียดออกซิเดชั่นในไต จากการทดลองพบว่าไซโคลสปอรินทำให้ความดันเลือดสูงขึ้น การทำหน้าที่ของไตลดลง ซึ่งประเมินจากการเพิ่มขึ้นของระดับยูเรียในพลาสม่า และการลดลงของอัตราการกรองของไต ไซโคลสปอรินกระตุ้นการทำงานของระบบประสาทซิมพาเตติกที่ไต ซึ่งประเมินจากการเพิ่มขึ้นของปริมาณนอร์อิพิเนฟรินและอิพิเนฟรินในไต และเหนี่ยวนำให้เกิดความเครียดออกซิเดชั่นเพิ่มขึ้น โดยการเพิ่มขึ้นของปริมาณ MDA ที่ไต และทำให้ปริมาณ reduced glutathione ที่ไตลดลง อย่างไรก็ตาม ไม่มีการเปลี่ยนแปลงของระดับ catalase ในไต โลซาทาน ซึ่งจะไปยับยั้งการทำงานของระบบระบบเรนินแองจิโอเทนซิน ส่งผลให้ความดันเลือดลดลง การทำหน้าที่ของไตดีขึ้น ปริมาณนอร์อิพิเนฟรินในไตลดลง และ ลดปริมาณ MDA ที่ไต จากผลการทดลองแสดงให้เห็นบทบาทของระบบเรนิน-แองจิโอเทนซิน ในการกระตุ้นการทำงานของระบบประสาทซิมพาเตติกที่ไต และ เหนี่ยวนำให้เกิดความเครียดออกซิเดชั่น ในหนูที่ถูกเหนี่ยวนำให้เกิดความเสียหายของไตด้วยไซโคลสปอริน ดังนั้นการยับยั้งระบบเรนิน-แองจิโอเทนซินน่าจะลดความเสียหายของไตในหนูที่ได้รับไซโคลสปอริน

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SUVAPORN PANASJAROEN : EFFECTS OF THE RENIN-ANGIOTENSIN BLOCKADE ON RENAL FUNCTION, RENAL NOREPINEPHRINE CONTENTS AND OXIDATIVE STRESS IN CYCLOSPORINE INDUCED NEPHROSIS RATS. THESIS ADVISOR : ASSOC. PROF. CHOLLADA BURANAKARL, Ph.D. THESIS CO-ADVISOR: SARINEE KALANDAKANOND-THONGSONG, Ph.D. 62 pp.

The objective of this study was to investigate whether of renin-angiotensin blockade improves renal function, renal norepinephrine contents and oxidative stress in cyclosporine (CsA) induced nephrosis rats. Rats were assigned into three groups and were treated as followed; group 1 (control group), receiving vehicle (propylene glycol) 1 ml/k.g./day for 28 days; group 2 (CsA group), receiving CsA 15 mg/kg./day, s.c. for 28 days; group 3 (CsA and losartan group), receiving CsA 15 mg/kg./day, s.c. with administration of losartan 10 mg/k.g./day orally for 28 days. Cyclosporine administration alone elevated mean arterial pressure, deteriorated renal function, as assessed by increased plasma urea nitrogen and decreased GFR. Cyclosporine stimulated renal sympathetic activity as assessed by increased renal norepinephrine content and induced oxidative stress, as indicated by increased renal MDA and decreased concentrations of renal reduced glutathione. No change was noted in renal catalase activity. Losartan, the AT_1 receptor antagonist markedly decreased mean arterial pressure, improved renal function, reduced renal norepinephrine contents and reduced level of renal MDA. These results clearly demonstrate that angiotensin II stimulates renal sympathetic activity and induced oxidative stress in CsA induced nephrosis rats. The therapeutic potential of AT_1receptor antagonist in ameliorating CsA-induced nephrosis rats is suggested.

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TABLE OF CONTENTS

	Page
ABSTRACT (THAI)	iv
ABSTRACT (ENGLISH)	v
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xii
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	4
- Pharmacology of cyclosporine	4
- Effects of cyclosporine on renal hemodynamic	6
- Effects of cyclosporine on sympathetic activity	7
- Effects of cyclosporine on renin-angiotensin system	8
- Effects of cyclosporine on oxidative stress	10
- Pharmacology of losartan	11
- Relationship between renin-angiotensin system and sympathetic activity	13
- Effect of renin-angiotensin system on oxidative stress	14
III MATERIALS AND METHODS	15
- Animals	15
- Experimental protocols	15
- Measurements of blood pressure and procedures for renal clearance study	18
- Determination of plasma and kidney catecholamine contents	19
- Determination of plasma, kidney and urine MDA	22
- Determination of kidney GSH	22
- Determination of kidney catalase activity	23
IV RESULTS	25
- Body weight	25
- Feed intake	26

CHAPTER **Page**

- Plasma creatinine and plasna urea nitrogen concentrations............. 26
- Effects of cyclosporine and losartan on renal hemodynamics.......... 29
- Effects of cyclosporine and losartan on mean arterial pressure and urine flow rate………………………………………………….. 31
- Effects of cyclosporine and losartan on plasma osmolality and plasma electrolyte concentrations... 33
- Effects of cyclosporine and losartan on osmolar clearance, free water clearance and fractional excretion of electrolytes.................. 33
- Effects of cyclosporine and losartan on urinary protein excretion and urinary protein creatinine ratio.. 34
- Effects of cyclosporine and losartan on urinary electrolyte excretion... 36
- Effects of losartan on cyclosporine induced oxidative stress........... 38
- Effects of cyclosporine and losartan on kidney and plasma catecholamine contents... 44

V. DISCUSSION.. 48

REFERENCES.. 54

BIOGRAPHY.. 62

LIST OF TABLES

PAGE

Table 4.1 Plasma creatinine and plasma urea nitrogen at day 0, day 14 and day 28 in all group.......... 27

Table 4.2 Glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR), filtration fraction (FF) and pack cell volume (PCV) in all group.......... 30

Table 4.3 Mean arterial pressure (MAP) and urine flow rate at day 28 of treatment in each group.......... 32

Table 4.4 The plasma osmolality (Posm), and plasma electrolyte concentrations in all groups at day 28 of treatment.......... 33

Table 4.5 Osmolality clearance (Cosm), free water clearance (CH_2O) and fractional excretions (Na^+, K^+, Cl^-) at day 28 in each group.......... 34

Table 4.6 Urinary protein excretion and urinary protein creatinine ratio (UPC ratio) at 0, 14 and 28 days of treatment in three groups.......... 35

Table 4.7 Urinary excretion of the electrolytes (sodium, potassium and chloride) in each group at day 0, day 14 and day 28.......... 37

Table 4.8 Urinary malondialdehyde excretion (nmol/day) at day 0, 14 and 28 after treatment.......... 38

Table 4.9 Urinary malondialdehyde creatinine ratio (nmol/ mg creatinine) at day 0, 14 and 28 after treatment.......... 39

Table 4.10 Effect of cyclosporine and losartan on reduced glutathione, catalase activity, kidney and plasma malondialdehyde.......... 41

Table 4.11 Plasma and kidney concentrations of norepiephrine, epinephrine and dopamine in each group.......... 44

LIST OF FIGURES

		PAGE
Figure 2.1	The chemical structure of cyclosporine..	5
Figure 2.2	The chemical structure of losartan..	12
Figure 4.1	Mean values for percent changes of body weight at days 7, 14, 21 and 28 in each group...	25
Figure 4.2	Daily mean values for feed intake in three groups of rats throughout the experimental period..	26
Figure 4.3	Mean values for plasma creatinine concentrations at day 0, day 14 and day 28 in each group..	28
Figure 4.4	Mean values for plasma urea nitrogen at day 0, day 14 and day 28 in each group..	29
Figure 4.5	Mean values of glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR) and filtration fraction (FF) in all groups..	31
Figure 4.6	Effect of CsA and cotreatment with losartan (LST) on mean arterial blood pressure...	32
Figure 4.7	Mean values of urinary protein excretion at 0, 14 and 28 days of treatment in three groups..	36
Figure 4.8	Mean values of urinary MDA excretion at day 0, 14 and 28.........	39
Figure 4.9	Mean values of urinary MDA creatinine ratio at day 0, 14 and 28..	40
Figure 4.10	Effect of cyclosporine (CsA) and losartan (LST) on kidney reduced glutathione...	41
Figure 4.11	Effect of cyclosporine (CsA) and losartan (LST) on kidney catalase...	42
Figure 4.12	Effect of cyclosporine (CsA) and losartan (LST) on kidney MDA concentration...	42
Figure 4.13	Effect of cyclosporine (CsA) and losartan (LST) on plasma MDA concentration...	43
Figure 4.14	Plasma concentrations of norepinephrine, epinephrine and dopamine in each group...	45

PAGE

Figure 4.15 Kidney concentrations of norepinephrine, epinephrine and dopamine in each group.. 45
Figure 4.16 Chromatograms of catecholamine extract from plasma................ 46
Figure 4.17 Chromatograms of catecholamine extract from kidney................ 47

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF ABBREVIATIONS

AII	Angiotensin II
AT_1 receptor	Angiotensin 1 receptor
BW	body weight
CAT	catalase
CsA	cyclosporine
DA	dopamine
DHBA	3,4-dihydroxy-benzyl-amine hydrobromine
E	epinephrine
ERBF	Effective renal blood flow
ERPF	Effective renal plasma flow
FE	Fractional excretion of electrolyte
FF	Filtration fraction
GFR	Glomerular filtration rate
GSH	Reduced glutathione
HPLC	high-performance liquid chromatography
LST	losartan
MAP	Mean arterial blood pressure
MDA	malondialdehyde
min	minute
NE	norepinephrine
PUN	Plasma urea nitrogen
RAS	Renin angiotensin system
ROS	Reactive oxygen species
RVR	Renal vascular resistance
SE	Standard error
TBARS	Thiobarbituric acid substances
UPC ratio	Urinary protein creatinine ratio

CHAPTER I

INTRODUCTION

Cyclosporine (CsA) is an immunosuppressive agent that can significantly improve survival rates and quality of life after organ transplantation. The immunosuppressive properties of cyclosporine have been proved to be useful in several diseases such as uveitis, psoriasis, systemic lupus erythematosus, rheumatoid arthritis and nephrotic syndrome (Remuzzi and Perico., 1995). However, the clinical usage of cyclosporine is often limited by chronic nephrotoxicity and hypertension. (Mason., 1989). A number of adverse effects of cyclosporine on renal function have been described, including severe impairment in glomerular filtration rate, reduction in renal blood flow related to afferent arteriolar vasoconstriction (Li et al., 2004), increased in renal vascular resistance, progressive renal failure, irreversible renal striped interstitial fibrosis and tubular atrophy and hyalinosis of afferent arteriole (Yang et al., 2002). Although cyclosporine is known to have significant nephrotoxic effects, the incidence of cyclosporine associated hypertension substantially exceeds that of the nephrotoxicity (Ciresi et al., 1992). Cyclosporine induced hypertension is associated with systemic vasoconstriction and increase efferent sympathetic nerve activity (Zhang and Victor, 2000).

The mechanisms underlying chronic cyclosporine nephrotoxicity are not fully understood. Several mechanisms have been proposed such as activation of the sympathetic system, stimulation of the intrarenal renin-angiotensin system, increased release of endothelin-1, dysregulation of nitric oxide, induction of transforming growth factor-beta1, stimulation of inflammatory mediators and increased reactive oxygen species (Elzinga et al., 2000; Li et al., 2004; Scherrer et al., 1990).

The current usage of cyclosporine may cause activation of the sympathetic nervous system (Lyson et al., 1993) via activation of renal afferent nerves (Hausberg et al., 2000). Likewise, enhanced sympathetic nerve traffic accompanies cyclosporine treatment in human plays an important role in the development of hypertension (Scherrer et al., 1990). Cyclosporine induced increase in renal vascular resistance is greatly attenuated, when the influence of sympathetic discharge is removed with

either renal denervation or the concurrent administration of alpha adrenergic blocker or the central sympatholytic drug (Scherrer et al., 1990). Several studies indicated that cyclosporine activates sympathetic nerve activity. Since renal nerve stimulation increases intrarenal angiotensin II formation, it is possible that cyclosporine induced activation of sympathetic nerve activity is involved in the augmentation of angiotensin II production (Dibona., 2000).

The intrarenal renin-angiotensin system (RAS) plays an important role in glomerular haemodynamics and kidney structure. Several evidences suggest an involvement of RAS in cyclosporine nephrotoxicity (Mason et al., 1991). Increased plasma renin activity and renin content in kidney tissues were observed in rats treated with long- term cyclosporine (Tufro-McReddie et al., 1993). Avdonin et al. (1999) reported that chronic cyclosporine administration upregulates AT_1 receptors in vascular and renal tissue. The renin-angiotensin system is a vasoactive factor that mediates renal vasoconstriction. In addition, both angiotensin II and cyclosporine cause an over-expression of TGF-β_1,a growth factor which has been implicated in the pathophysiology of renal fibrosis (Sun et al., 2005).

Activation of the renin-angiotensin system in the failing kidneys may be responsible for the increased sympathetic nerve discharge. Ligtenberg et al. (1999) reported the decreased muscle sympathetic nerve activity in patients with chronic renal failure after treatment with angiotensin-converting enzyme inhibitor.

Chronic ischemia caused by CsA is believed to be associated with reactive oxygen species (ROS) and lipid peroxidation (Tariq et al., 1999). The products of lipid peroxidation were found to be extremely high in kidney tissue of rats treated with cyclosporine (Suleymanlar et al., 1994). Zhong et al. (1998) showed that CsA-induced sympathetic nerve activation and reactive oxygen species (ROS) production in the kidney could be prevented by renal denervation. Therefore, it is possible that CsA increases ROS production by increasing renal sympathetic nerve activity, resulting from vasoconstriction. Furthermore, it was recently shown that ROS production induced by elevated angiotensin II levels contributes to the development of cyclosporine-induced hypertension (Nishiyama et al., 2003)

We hypothesized that, (1) cyclosporine increases intrarenal norepinephrine concentration with impaired renal function, (2) cyclosporine treatment causes renal damage, which can be detected by changes of lipid peroxidation and (3) losartan

improves renal function and reduce oxidative stress with concomittant reduction in renal norepinephrine content in rats treated with cyclosporine.

Accordingly, the aims of this study are first, to study the relationship between AT1 receptor antagonist (losartan) and renal catecholamine concentration. Second, to determine the effect of losartan on renal function and oxidative stress in cyclosporine induced nephrotoxicity.

CHAPTER II

LITERATURE REVIEW

Pharmacology of cyclosporine

Cyclosporine is a cyclic polypeptide consisting of eleven amino acids (Figure 2.1). It is an immunosuppressive agent produced by the soil fungus *Beauveria nivea*. The chemical name of cyclosporine is [*R*-[*R**, *R**-(*E*)]]-cyclic(L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl-3-hydroxy-*N*,4-dimethyl-L-2-amino -6- octenoyl -L- α -amino-butyryl-*N*-methylglycyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl). The cyclosporine compound is soluble only in organic solvents or lipids, but insoluble in water. It is distributed largely in the extravascular space with a mean apparent volume of distribution of 3.5 l / kg. With the blood distribution depends on the active ingredient concentration: 33-47 % is found in plasma, 4 - 9 % in lymphocytes, 5 – 12 % in granulocytes and 41 - 58% in erythrocytes. In plasma, approximately 90% is bound to proteins. Cyclosporine metabolism is in the liver using cytochrome P-450. Elimination is primarily biliary, with only 6% of an oral dose excreted in the urine and less than 1% is excreted as unchanged drug (Li et al., 2004; Kahan, 1989; Schreiber and Crabtree, 1992).

Experimental evidence suggests that the effectiveness of cyclosporine is due to specific and reversible inhibition of immunocompetent lymphocytes in the G_0- or G_1-phase of cell cycle (Rusnak and Mertz, 2000). Cyclosporine can interact with cytoplasmic membrane and activate the intracellular calcium pathway (Erlanger, 1992). One study proposed that cyclosporine enters the cell passively and binds to a specific family of receptors know as cyclophillins. This drug receptor complex inhibits IL-2 production by cyclosporine stops proliferation and activation of T lymphocytes (Busauschina et al., 2004). The T-helper cell is the main target, although the T-suppressor cell may also be suppressed.

Figure 2.1 The chemical structure of cyclosporine

Clinical usage of cyclosporine

Cyclosporine is used to prevent or treat organ rejection in transplant patients. It has improved allograft survival and quality of life for solid-organ transplant recipients. More recently cyclosporine has proved beneficial in the treatment of many autoimmune disease including uveitis, psoriasis, systemic lupus erythematosus, rheumatoid arthritis and various forms of glomerulonephritis (Remuzzi and Perico, 1995).

However, several adverse effects have been reported, including nephrotoxicity, hepatotoxicity and neurotoxicity, hypertension, increased risk of cardiovascular events, tremor, hypertrichosis, gastrointestinal disturbances and gingival hypertrophy (Li et al., 2004).

Effects of Cyclosporine on renal hemodynamic

Kidney dysfunction is the main complication of cyclosporine treatment. About 30% of patients treated with cyclosporine have moderate to severe kidney damage (Barros et al., 1987). This effect is most easily detected in humans by increases in serum urea or creatinine concentrations. Chronic cyclosporine nephrotoxicity is characterized by renal afferent arteriole vasoconstriction, decreased glomerular filtration rate, decreased renal blood flow and increased renal vascular resistance (Murray et al., 1985). This was associated with hypertension. There are many suggested mediators responsible for cyclosporine-induced vasoconstriction, including angiotensin II, sympathetic nervous system activation, reduction of vasodilator prostaglandins and nitric oxide and increased vasoconstrictor, endothelin, thromboxane A_2, platelet derived growth factor (Andoh et al., 1997).

In addition, cyclosporine has been shown to impair renal autoregulatory mechanisms (Devarajan et al., 1989). Laskow et al. (1990) showed that salt depletion decreased GFR in cyclosporine treated patients.

Acute renal dysfunction due to cyclosporine is reversible since it involves renal hemodynamic dysfunction but not associated with any permanent histologic change (English et al., 1987). On the other hand, chronic cyclosporine nephrotoxicity may progress to an irreversible renal lesion characterized by striped interstitial fibrosis, tubular dilatation and atrophy, hyalinosis of the afferent arteriole and arteriopathy (Burdmann et al., 1995; Young et al., 1995). The arteriopathy consisted initially of eosinophilic granular transformation of smooth muscle cells comprising afferent hilar glomerular arterioles, and progressed to foci of smooth muscle cell vacuolization and accumulation of discrete hyaline deposits in vessel walls. The marked histological changes were prominent in the outer cortex and medullary region of the kidney.

Cyclosporine affects both the reabsorption and the secretory functions of the renal tublues. Previous study suggested that effects of cyclosporine may be secondary to sodium retention with central volume expansion (Curits et al., 1988). Ciresi et al. (1992) demonstrated that chronic cyclosporine administration results in active sodium retention with associated activation of the renin-angiotensin system and suppression of circulating atrial natriuretic factor (ANF). Futhermore, there was an attenuated

natriuretic and diuretic responses to the acute challenge of intravenous volume expansion in the cyclosporine treated rats which resulted from enhanced tubular reabsorption of sodium. Urine volume was generally unchanged or only marginally increased in rats given cyclosporine experimentally. Urine osmolality was unaltered or only marginally decreased in rats treated with cyclosporine (Dieperink et al., 1986; Mason et al., 1989; Whiting and Simpson, 1988). Nishiyama et al. (2003) reported that urinary protein excretion was not altered in hypertensive rats induced by cyclosporine.

Chronic ischemia caused by cyclosporine is believed to be associated with reactive oxygen species (ROS) and lipid peroxidation (Li et al., 2004). In the rats model, cyclosporine caused an elevation of lipid peroxidation in kidney tissue (Suleymanlar et al., 1994). Recent studies clearly demonstrated that cyclosporine induced oxidative stress could play pivotal role in producing structural and functional impairment of kidney (Tariq et al., 1999).

Effects of cyclosporine on sympathetic activity

The sympathetic nervous system modulates cardiac output and peripheral vascular resistance, which determines arterial blood pressure. An abnormality in the sympathetic nervous system might contribute to the development or maintenance of hypertension. Activation of the sympathetic activity and activation of the intrarenal renin-angiotensin system have all been implicated in the pathogenesis of chronic cyclosporine nephrotoxicity (Scherrer et al., 1990; Li et al., 2004). Indeed, sympathetic nervous system hyperactivity has been postulated to play a major role in the intense intrarenal vasoconstriction and hypertension provoked by cyclosporine (Elzinga et al., 2000).

Murray et al.(1985) showed the possibility that renal vasoconstriction in response to cyclosporine might be mediated by increased levels of circulating catecholamines or increased renal nerve activity. By administered the α - adrenergic blocker, phenoxybenzamine, to rats infused with cyclosporine demonstrated an increase in renal blood flow and a decrease in renal vascular resistance. However phenoxybenzamine did not affect renal blood flow or vascular resistance in control animals. Therefore, renal vasoconstriction following cyclosporine infusion was

mediated through increased renal nerve activity. Likewise, enhanced sympathetic nerve traffic accompanies cyclosporine treatment in human play an important role in the development of hypertension (Scherrer et al., 1990).

Elzinga et al. (2000) reported that renal denervation did not alter the lesion of cyclosporine-induced renal injury in rats maintained on a low salt diet. These results suggest that mechanisms other than renal sympathetic nerve hyperactivity are responsible for the development of chronic cyclosporine nephropathy. However, in these studies they did not use pharmacologic sympathetic blockade. They were unable to detect any differences between the innervated and denervated kidney which may be due to the possibility of α - adrenergic receptor upregulation or enhanced sensitivity in the denervated kidney. Several studies indicate that cyclosporine activates sympathetic nerve activity (Ryuzaki et al., 1997; Zhong et al., 1998). Since renal nerve stimulation increases intrarenal angiotensin II formation, it is possible that cyclosporine induced activation of sympathetic nerve activity is involved in the augmentation of angiotensin II production (Yamaguchi et al., 2000). Likewise, renal ischemia has been elevated AII and suppressed brain nitric oxide all stimulate sympathetic activity (Koomans et al., 2004).

Furthermore, cyclosporine induced hypertension seems to be mediated by synapsin, stored in the renal afferent nerve ending, because in the synapsin knockout mice, cyclosporine failed to induce hypertension (Zhang et al., 2000).

Effects of cyclosporine on renin-angiotensin system (RAS)

Activation of the RAS, especially the intrarenal RAS plays an essensial role in the pathogenesis of chronic cyclosporine nephrotoxicity. The hypertensive effects of cyclosporine was mostly due to the effect of angiotensin II on vasoconstriction, since giving cyclosporine in the presence of an ACE inhibitor prevented blood pressure elevation in conscious dog (Ouisuwan and Buranakarl, 2005). However, the mechanism of activation of RAS in this complication is still unknown. One accepted hypothesis is that cyclosporine can increase renin release from juxtaglomerular cells. Myers et al. (1988) found the extreme elevation of plasma prorenin and total renin concentration, together with prominent hyperplasia of juxtaglomerular apparatus

(JGA). In man, a corresponding JGA hyperplasia has been observed early after starting cyclosporine therapy following heart transplantation (Mason et al., 1991).

Since the plasma renin activity is a rate-limiting step in angiotensin II production, cyclosporine may lead to increased angiotensin II levels through renin. In addition, both rat and human kidney show a high concentration of AII receptor in the inner zone of the outer medulla, particularly in longitudinal bands paralleling the vasa recta bundle, suggesting that regional regulation of medullary blood flow is mediated by angiotensin II (Lee, 1997). Recently, these receptor were characterized as being of the AT_1 subtype (Sechi et al., 1992). Avdovin et al. (1999) showed that pretreatment with cyclosporine for 24 hours increased [^{125}I] angiotensin II binding in vascular smooth muscle cells without changing its affinity, suggesting an enhancement of angiotensin II receptor expression by acute cyclosporine treatment. Moreover, long term treatment with cyclosporine increased protein expression of AT_1 receptor in the aorta (Nishiyama et al., 2003). These observations are in accordance with previous studies showing that mRNA expression of AT_1 receptors in aortic vascular smooth muscle cells and endothelial cells were significantly increased in cyclosporine induced hypertensive rat. (Iwai et al., 1993)

Angiotensin II has profound effects on blood pressure and renal function. Angiotensin II increase blood pressure directly by direct vasoconstriction both peripherally and at the renal vasculature, especially at afferent arterioles, causing reduced renal blood flow (Yuan et al., 1990). One study found that blockade of RAS with an angiotensin II receptor antagonist or ACE inhibitor strikingly prevented cyclosporine-induced interstitial fibrosis without improving renal function parameter (Burdmann et al., 1995). These results strongly suggest that the mechanisms promoting the interstitial scarring in chronic cyclosporine nephrotoxicity are angiotensin II dependent and can be dissociated from those causing glomerular and tubular dysfunction (Burdmann et al., 1995). On the other hand, the RAS may also induce renal injury nonhemodynamically via stimulation of tubulointerstitial inflammation, TGF-β1, vascular endothelial growth factor, and increase renal cell apoptosis (Li et al., 2004). The previous study has been reported that TGF-β1 play an important role in fibrosis arising from chronic cyclosporine nephrotoxicity (Shihab et al.,1997). Sun and coworkers (2005) showed that increased TGF-β1 mRNA expression in cyclosporine-induced nephrotoxicity was significantly reversed by AT_1

receptor antagonist, losartan co-treatment. However evidence of AT_1 blockade can improve renal dysfunction was demonstrated that plasma creatinine levels were significantly increased in cyclosporine induced hypertensive rats, suggesting cyclosporine induced glomerular dysfunction (Haugan et al., 2000). Padi and Chopra (2002) also observed that treatment with candesartan (AT_1 receptor antagonist) significantly decreased plasma creatinine levels in cyclosporine treated rats. These results indicated that cyclosporine induced glomerular dysfunction was ameliorated by treatment with candesartan.

Effects of cyclosporine on oxidative stress

Chronic administration of cyclosporine has been shown to increase significantly renal vascular resistance and decreased renal blood flow causing hypoperfusion and ischemia (Mason, 1989). Renal ischemia, following impaired tissue perfusion, results in the rapid breakdown of tissue accompanied by excessive generation of oxygen-free radical (OFR) (Tariq et al., 1999). Reactive oxygen species (ROS) or oxygen-free radicals (OFR) are O_2 molecules with an unpaired electron and include superoxide anion (O_2^-), H_2O_2 and hydroxy ion (OH) (Campese et al., 2004). Some evidence suggested that ROS production is the central process in the pathophysiology of cellular toxicity by many agents and by ionizing radiation (Suleymanlar et al., 1994). The production of ROS by cyclosporine may be due to the action of cyclosporine as an uncoupler and inhibitor of the mitochondrial electron transport system or during its metabolism by cytochrome P450 (Hagar et al., 2006).

Zhong et al. (1998) have shown that cyclosporine administration results in excess local production of hydroxy radical, leading to lipid peroxidation and nephrotoxicity. Cyclosporine increased renal lipid peroxides, measured as TBARS, indicating increased ROS activity and oxidative stress (Padi and Chopra, 2002). Lipid peroxidation (LPO) seems to be the main mechanism of free radical toxicity. Cellular membranes, especially membranes of mitochondria and endoplasmic reticulum, are very susceptible to the lipid peroxidative deterioration. The lipid peroxidation can be assessed by measuring relatively stable lipid hydroperoxides (LPH) such as malondialdehyde (MDA), conjugated diens (CD) and Schiff bases (SB) in biological

materials (ie, plasma, urine and tissue). As a result, measurement of lipid hydroperoxide levels may be a useful index to determine the severity of free-radical induced cell injury (Suleymanlar et al., 1994).

The main detoxifying system for lipid peroxides is glutathione. Reduced glutathione (GSH) is a predominant non-protein thiol in virtually all cell types which plays an important role in the detoxification of xenobiotics. Glutathione retains cellular metabolic functions and integrity by stabilizing cell membrane (Brezis et al., 1983). A decrease in glutathione concentration in the renal cortex is a common biochemical response to many nephrotoxic agents. The treatment of rats with cyclosporine produced a significant increase O_2^-, H_2O_2 ,OH^- radicals production and increase in renal LPH and MDA, and decrease in glutathione level (Parra et al., 1998; Suleymanlar et al., 1994; Tariq et al., 1999).

Overproduction of reactive oxygen species (ROS) and lipid peroxidation are pathogenic factors involved in chronic cyclosporine induced renal injury and antioxidant agents may be therapeutic. Parra et al. (1998) showed that pretreatment with vitamin E prevented the renal impairment induced by cyclosporine, probably through the scavenger effect of the vitamin on the formation of oxygen free radicals, and by inhibition of lipid peroxidation.

The previous study showed that cyclosporine-induced sympathetic nerve activation and ROS production in the kidney which were prevented by renal denervation (Nishiyama et al., 2003). Therefore, it is possible that cyclosporine increased ROS production via increased renal sympathetic nerve activity.

Pharmacology of losartan

Losartan potassium is a selective angiotensin II type-1 receptor (AT_1) antagonist. Losartan, a non-peptide molecule, is chemically described as 2-butyl-4-chloro-1-[[2´-(1*H*-tetrazol-5-yl)[1,1´-biphenyl]-4-yl]methyl]-1*H*-imidazole-5-methanol monopotassium salt. The chemical formula is $C_{22}H_{22}ClKN_6O$. The chemical structure is shown in figure 2.2. Losartan is a white to off-white free-flowing crystalline powder. It is freely soluble in water. Following oral administration, losartan is well absorbed and undergoes substantial first-pass metabolism by cytochrome P450 enzyme. Mean peak concentration of losartan occur at about one

hour. The terminal half-life of losartan is about 2 hours and of the metabolite is about 6-9 hours. Both losartan and its active metabolite are highly bound to plasma proteins. Studies in rats indicate that losartan crosses the blood-brain barrier poorly (Ye et al., 2002)

Figure 2.2 – the chemical structure of losartan.

The renin-angiotensin system (RAS) is a bioenzymic cascade that plays an integral role in cardiovascular homeostasis by influencing vascular tone, fluid and electrolyte balance and the sympathetic nervous system. The RAS was reviewed as a circulating endocrine system, whereby renin released from the juxtaglomerular cell of the kidney cleaves the liver-derived macroglobulin precursor angiotensinogen, to produce the inactive decapeptide angiotensinogen I, which is then converted to the active octapeptide angiotensin II by angiotensin-converting enzyme (ACE) within the pulmonary circulation .In addition to the circulating RAS, there is evidence to indicate that many tissues, including the vasculature, heart, kidney and brain have local RAS (Alpern, 1997; Campbell, 1987; Johnson et al., 1992). The action of angiotensin II are mediated by specific heterogenous populations of angiotensin II receptors. Angiotensin II is known to interact with at least two distinct angiotensin II receptor subtypes, designated AT_1 and AT_2 (Dinh et al., 2001). Virtually all the known biological actions of angiotensin II, including vasoconstriction, release of aldosterone, stimulation of sympathetic transmission and cellular growth, are exclusively mediated by the AT_1 receptor.

Relationship between renin-angiotensin system and sympathatic activity

Recent study has confirmed the increased sympathetic activation and high-normal serum norepinephrine concentrations in heart-transplanted patients or patients suffering from myasthenia gravis, recieving cyclosporine (Scherrer et al., 1990). Although the precise nature of this signal is not known, at least two possibilities might be considered. First, the failing kidney released humoral substance, such as renin, that might lead to central activation of sympathetic outflow (Converse et al., 1992). Angiotensin II can stimulate sympathetic nerve activity by a direct effect on the vasomotor center in the brainstem and AII also facilitates sympathetic neurotransmission at the adrenergic nerve terminal by increased release and decreased uptake of norepinephrine. Conversely, AII enhanced sympathetic nervous activity by its effect on blood pressure, via the baroreflex (Koomans et al., 2004; Ouisuwan and Buranakarl., 2005). A second way by which the kidneys can command the brain to increase sympathetic outflow by increased renal afferent nerve activity with injured or ischemic kidneys (Blankestjin et al., 2000; Johansson et al., 1999).

One previous study showed that losartan reduces blood pressure in CRF rats largely through inhibition of central SNS activity. Although the intravenous dose of losartan required to inhibit SNS activity was far greater than that necessary to achieve the same results when given intracerebroventricularly, qualitatively the results were the same. This suggests that, even when given intravenously, losartan may penetrate into the brain in sufficient amounts to inhibit central SNS activity (Ye et al., 2002).

Effect of renin angiotensin system on oxidative stress

The mechanisms responsible for progressive nature of AII induced hypertension are multifarious. However, recent studies have implicated a role of ROS in the pathogenesis of AII-dependent hypertension (Romero and Reckelhoff., 1999). Nishiyama et al. (2003) demonstrated that cyclosporine-induced hypertension is associated with elevated AII and ROS formation in rats. In addition, AT_1 receptor blockade prevented increases in ROS levels and development of hypertension induced by long-term treatment with cyclosporine. Recently, it has been demonstrated that angiotensin II stimulates NAD(P)H oxidase-dependent superoxide production in kidney, proximal tubular cells and vascular smooth muscle cells which is transduced through AT_1 receptors.The superoxide produced on angiotensin II stimulation is rapidly converted to other ROS.

Therefore the effect of cyclosporine-induced nephrotoxicity may be due to enhanced sympathetic activity and oxidative stress via stimulate AII. Thus, by using angiotensin II type-1 receptor antagonist (losartan) we expected the improved renal function and reduced oxidative stress along with a decline of sympathetic activity in nephosis rats induced by cyclosporine.

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats weighing between 200 to 250 g were obtained from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand. The animals were housed individually and were maintained at 25 $\pm$ 2ºc under a controlled light and dark cycles (L:D = 12:12). All rats were fed *ad libitum* with standard rat chow (CP, Thailand) and allowed free access of tap water.

All procedures were done with the approval of the Animal Use Commitee, Faculty of Veterinary Science, Chulalongkorn University.

Experimental protocol

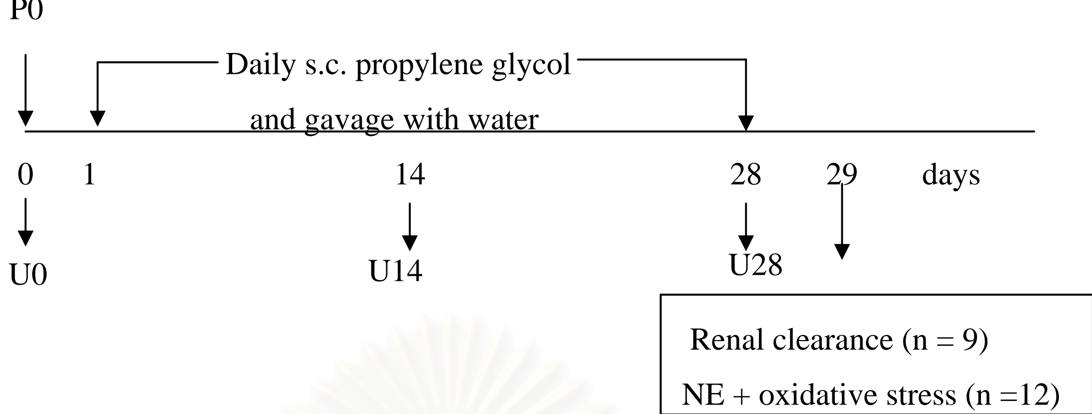
After a seven day adaptation period in the laboratory condition, the animals were divided into three groups.

Group 1 (control, n = 21), rats were received a daily subcutaneous injection of propylene glycol (1ml / kg.) and gavage with water (1 ml / kg.) as a control for 28 days.

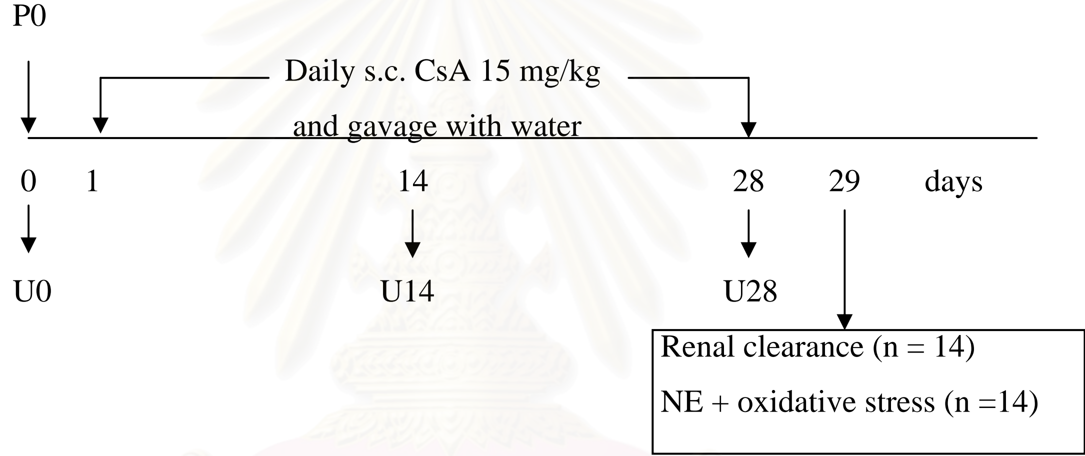
Group 2 (Cyclosporine, n = 28), rats received a daily subcutaneous injection of cyclosporine (Sandimmun®, Novartis Pharma AG, Basle, Switzerland) at the dose of 15 mg/kg b.w. and gavage with water (1 ml / kg.) for 28 days (Burdmann et al., 1995).

Group 3 (Cyclosporine + Losartan, n = 21), rats received a daily subcutaneous injection of CsA at the dose of 15 mg/kg b.w. and losartan (Cozaar®, Merck Sharp & Dohme LTD., England) at the dose of 10 mg /kg b.w. by gavaged for 28 days. This dosage and route of administration for losartan is selected because it has previously shown to significantly block angiotensin II receptors (Burdmann et al., 1995).

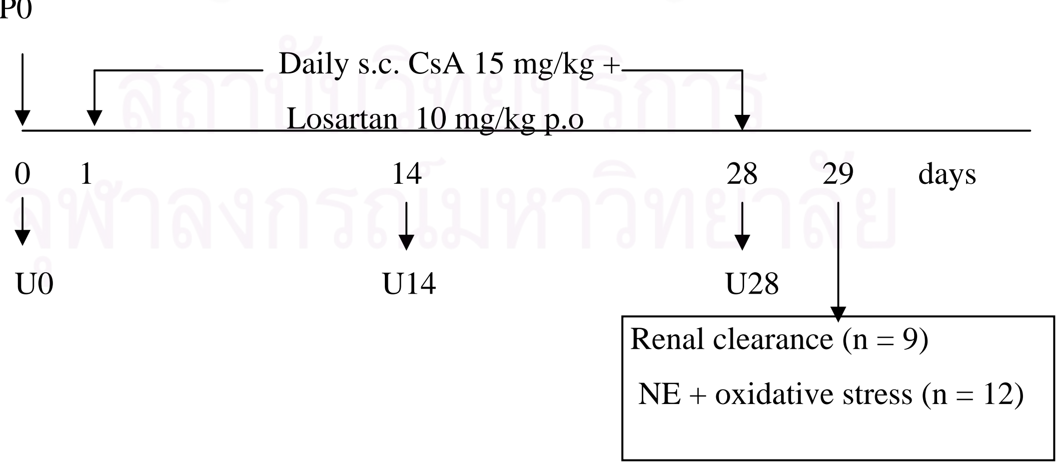
1. Control group



2. Cyclosporine



3. Cyclosporine + Losartan



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Body weight and feed intake were recorded everyday throughout the experimental period. At day 0, 14 and 28, the rats were placed in individual metabolic cage for 24-hr urine collections for measurement of urine volume, urinary protein and electrolyte concentrations (Na^+, K^+, Cl^-), osmolarity and malondialdehyde (MDA) concentrations. During this period, the food was withheld with free access of water**.** Plasma was collected by cutting tip of rat's tail vein for measurement of plasma urea nitrogen (PUN) and creatinine. The renal clearance was performed in 32 animals from three groups on day 29. Other animals were euthanized without renal function study to evaluate catecholamine contents and oxidative stress in the kidney.

Blood Sampling and Tissue preparation

Renal clearance study was performed in 9, 14 and 9 rats, respectively on day 29th. After completion of renal clearance study, 4 ml of blood (Pa) was collected from the right femoral artery in tubes containing heparin to measure creatinine, PUN, osmolality and electrolytes (Na, K, Cl).

In each group, other rats were anesthetized with halothane (Rhodia Organique Fine LTD, UK.). Blood was collected from cardiac puncture to measure plasma MDA and norepinephrine. The right kidney was removed immediately, rinsed free of blood with normal saline, cut in half, and placed in ice-cold saline. Thereafter, the outer cortex was cut to collect fragments weighing about 100 mg and placed in vials containing 500 μL of 0.4 mol/L $HClO_4$. The samples were stored at -80°C until assayed for catecholamines. Catecholamine content was determined by reversed-phase HPLC detection (Vieira-Coelho et al., 1999). The left kidney was removed immediately and stored at -80°C for the analysis of reduced glutathione, MDA and catalase activity.

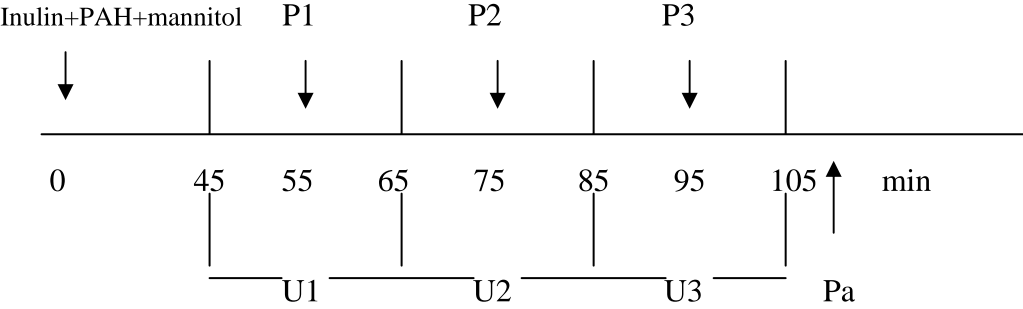
Surgical preparation for renal clearance study

Rats were anesthetized with zolazepam and tiletamine (zoletil®, VIRBAC Laboratories, Carros, France, 40 mg/kg b.w.) and xylazine (Seton®, Laboratorios Calier, S.A., Spain, 5 mg/kg b.w.,) via an intraperitonial injection. The trachea was cannulated with polyethylene tubing (PE 240) for aspirating secretion and used as an artificial airway. A catheter (polyethylene tubes; PE50) was inserted into the femoral artery for measuremant of arterial blood pressure and also for blood collection. The femoral vein was cannulated with PE50 catheter for infusion of inulin and para-aminohippurate (PAH) solution. The abdominal midline incision was performed and urinary bladder was located while the PE 250 catheter was inserted for urine collection. Urine samples were collected into a pre-weighed eppendorf.

Measurements of blood pressure and procedures for renal clearance study

The arterial blood pressure and heart rate were monitored by connecting the arterial catheter to a pressure transducer with a Grass polygraph recorder. Blood pressure was recorded throughout the period of renal function study.

Renal clearance study was started by infusing a mixture of 1% inulin, 0.2% PAH and 6% mannitol in normal saline at the rate of 1 ml/h per 100 g body weight continuously for 45 minutes to stabilize plasma inulin and PAH concentrations. After equilibration period, three times of urine collection (U_1, U_2, U_3) along with arterial blood sampling at midpoint of urine collection (P_1, P_2, P_3) were performed. Bovine serum albumin (6%) was administered after blood collection at the same volume to replace blood loss. Each urine collection period was 20 minutes. Urine volume was measured from the weight changes of pre-weighed eppendorf. Blood sample was collected for determination of packed cell volume. Plasma and urine were kept at 4°C for analyses of inulin and PAH concentrations.



P1 + P2 +P3 : inulin, PAH, PCV

U1 + U2 + U3 : inulin, PAH

Pa : creatinine, BUN, osmolality and electrolyte (Na, K,Cl)

Determination of blood, urine and kidney samples

Plasma urea nitrogen, plasma and urinary creatinine concentrations were measured by automate (Humalyzer 2000 Human, H.E. supply LTD. TART.). The sodium and potassium concentrations in both urine and plasma were determined by flame photometer (Flame photometer 410C, Ciba Corning Diagnostic Instruments, Halstead, USA). Plasma and urinary chloride concentrations were determined by chloridometer (Chloride Analyzer 925, Ciba Corning Inc., USA.). Urine and plasma osmolarity were measured using an osmometer (Osmometer 3D3, Advanced instruments, Inc., Norwood, MA, USA). The fractional excretion of electrolytes were calculated by a standard formula. The inulin concentration was determined by the Antrone method as described by Young and Raisz (1952). The PAH concentration was determined by the method of Brun (1951).

Determination of plasma and kidney catecholamine contents

Extraction procedure for plasma norepinephrine and epinephrine

Extractions were performed according to previously published procedures by Anton and Sayre (1962). One ml of the plasma was placed in a 3-ml column containing frit (Alltech Associates Inc., Deerfied, Il, U.S.A.) along with 20 mg of

acid-activated alumina (Sigma, St. Louis, Mo), 1-ml of 1.5 M Tris, pH 8.8; (in order to adjust pH) and 50 µl of DHBA (5 pg), as an internal standard. NE, E and DHBA were allowed to absorb to an acid-activated alumina by gentle mixing on a horizontal shaker for 30 min. The absorbed alumina were then washed three times with ice-cold ultrapure water and centrifuged at 3000 g, at 4ºC for 3 min to remove excessed water. NE, E and DHBA were eluted from the alumina, following the addition of 100 µl 0.1 M PCA (Sigma), suspended by vortex-mixing for 20 min and centrifuged at 3000-x g, at 4ºC for 5 min. The extracts were collected and saved for injection into the HPLC system. All samples from each animal were extracted twice to provide the data of E and NE in duplicate.

Extraction procedure for kidney norepinephrine and epinephine

Kidneys were processed and assayed for norepinephrine and epinephine as described by Eldrup and Richter (2000). Briefly, kidney cortex previously collected and preserved was homogenized in 2 ml of ice-chilled 0.6 M perchloric acid containing 1.7 mg/ml EGTA, 1.1 mg/ml reduced glutathione, 60 µl of 10 mM $Na_2S_2O_3$ and 200 µl of DHBA (5 pg), as an internal standard. After centrifugation for 15 min at 2,500 g at 4ºC. One ml of the supernatant was adjusted to pH 8.7 with 1 ml of 1M Tris. NE, E and DHBA allowed to absorb to an acid-activated alumina by gentle mixing on a horizontal shaker, for 30 min. The absorbed alumina were washed three times with ice-cold ultrapure water and centrifuged at 3000 g, at 4ºC for 5 min to remove excesses water. NE, E and DHBA were eluted from the alumina, following the addition of 120 µl 0.2 M PCA , suspended by vortex-mixing for 20 min and centrifuged at 3000 g, at 4ºC for 3 min. The extracts were collected and saved for injection into the HPLC system. All samples from each animal were extracted twice to provide the data of E and NE in duplicate.

Chromatographic instrumentation

An HPLC system with an electrochemical detector, a glassy carbon working electrode and amperometric control (Bioanalytical systems, West Lafayette, IN, USA.) was used to measure the concentration of E, NE and DHBA. A Shimadzu Model LC-10 AD pump (Kyoto, Japan) was connected to a Rheodyne (Cotati, CA, USA.) injector, equipped with a 20 µl fixed loop and a 15-cm spherisorb®column, packed with 5-µm particles. The mobile phase solution was composed of 1.5 mM heptane sulfonate, 100 mM NaH_2PO_4, 1 mM Na_2EDTA and 4% methanol, adjusted to pH 4.1 with saturated citric acid. The mobile phase was filtered through a 0.22-µm filter, degassed by ultrasonic agitation and pumped at a flow-rate of 0.8 ml min^{-1}. The amperometer was set at a positive potential of 0.700 V with respect to the Ag/AgCl reference electrode, with a sensitivity of 0.2 nA. The extract (40 µl) from the plasma and kidney samples were injected into the HPLC-EC system to separate NE, E and DHBA. Data were collected and analyzed by Delta 5.0 software (Digital Solutions, Margate, QLD, Australia).

Analytical procedures

Standard solutions at different concentrations were injected into the HPLC system. The retention time was evaluated by injecting both standard catecholamine individually and by the injection of a standard mixture. The recovery of NE, E and DHBA after alumina extraction was calculated from the peak area before and after extraction. Standard solutions of the same concentration were injected repeatedly everyday for several days to verify the repeatability of the assay.

To obtain plasma calibration curves, plasma samples were pooled seperately. Several amounts of NE and E with a fixed amount of DHBA (as an internal standard) were added to 1 ml pooled plasma. The mixtures with different concentrations of standard were then treated similarly to the plasma samples. The absolute level of catecholamine was calculated as the percentual ratio between the peak areas of the catecholamines and the corresponding internal standard, after alumina extraction, to

yield a plasma calibration curve, after subtraction from the baseline endogenous NE and E. The levels of NE and E were presented as a mean ± S.E.

Determination of kidney, plasma and urine MDA

Malondialdehyde (MDA), an indirect index of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS). TBARS were determined by a method slightly modified from the method of Ohkawa et al. (1979). Briefly, renal cortex was weighed and properly minced. The 250 µl of tissue homogenate prepared in 1.15% potassium chloride, 15 µl of 10 mM butylated hydroxytoluene, 100 µl of 8.1 % sodium dodecyl sulfate, 750 µl of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 750 µl of 0.5% thiobarbituric acid were added. The mixture was made up to 2 ml with ultrapure water and heated at 95ºc for 60 minutes. After cooling with tap water, 2 ml n-butanol-pyridine (15:1, v/v) was added and centrifuged at 600g for 10 minutes. The upper organic layer was take out and its absorbance was measured at 532 nm by spectophotometer. Malondialdehyde tetraethylacetal was employed as the standard. MDA values were expressed as nmol of TBARS per milligram protein. The tissue protein was determined using Lowry method.

To determine plasma MDA concentration, 250 µl of plasma was employed with MDA assay describes above. Urine MDA was obtained using 250 µl of urine.

Determination of kidney GSH

Reduced glutathione (GSH) was determined by using a modification method of Beutler et al. (1963). Renal cortex tissues (0.2 g) were homogenized in 1.8 ml of 100 mM KCl plus 0.003 M EDTA. The homogenates were centrifuged at 600 g for 10 minutes. The 500 µl of supernatant was added to 750 µl metaphosphoric acid, and particulate debris was removed by centrifugation at 3,000 g for 10 minutes. The reaction mixture contained 0.5 ml supernatant, 2.0 ml of 0.2M phosphate buffer and 0.25 ml 0.04% 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB). The absorbance was determined at 412 nm. The results were expressed in nmol GSH per milligram protein.

Determination of kidney catalase activity

Kidney catalase activity was measured using the modification method from Aebi et al. (1983). Renal cortex (0.2g) was homogenized with ten part of enzyme dilution buffer (50 mM phosphate buffer, pH 7.0, containing 0.1% Triton X-100) weight by volume (w/ v). The 100 µl of the supernatant was added to 1.9 ml of phosphate buffer to a quartz cuvette and mixed by inversion. Start the reaction by addition of 1 ml of 30 mM H_2O_2 and mixed well with a plastic paddle. The change in absorbance was read at 240 nm every 30 sec for 1-2 min by using UV-VIS spectrophotometer. Catalase activity was expressed as unit per milligram protein.

Calculation of blood pressure and renal function

Mean arterial blood pressure = DP+1/3(PP)

Glomerular filtration rate (GFR) = $U_{in}V / P_{in}$

Effective renal plasma flow (ERPF) = $U_{PAH}V / P_{PAH}$

Effective renal blood flow (ERBF) = ERPF x 100 / (1-PCV)

Filtration fraction (FF) = GFR x 100 / ERPF

Renal vascular resistance (RVR) = MAP / ERBF

Urinary excretion of substance = U x V

Fractional excretion of electrolyte (FE) = $\frac{UeV / Pe \times 100}{GFR}$

Cosm = UosmV / Posm

CH_2O = V - Cosm

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Statistical analysis

All data are expressed as mean $\pm$ standard error. To compare between groups, one-way analysis of variance (One-way ANOVA) was used and followed by Student-Newman-Keuls. In the case of the normality test failed, the one-way analysis of variance on rank was used and followed by Dunn's test. To compared within group at different time point, one way repeated measures ANOVA was used and followed by Student-Newman-Keuls. In the case of the normality test failed, one way ANOVA with repeated measures on ranks was used and post hoc analysis with Student-Newman-Keuls or Dunn's. Differences between means were considered statistically significant at $P<0.05$. Sigma-stat solfware was used for statistical analysis.

CHAPTER IV

RESULTS

Body weight

The percent changes of body weight at days 7, 14, 21 and 28 in each group are presented in figure 4.1. Means weight were similar before treatment. The average percent change of body weight at day 28 of group 1 (control group), group 2 (CsA treatment) and group 3 (CsA cotreatment with losartan) were 58.23 $\pm$ 2.3 , 37.4 $\pm$ 2.4 and 40.88 $\pm$ 1.33 % , respectively. At days 7, 14, 21 and 28 of treatment, the percent changes of body weight of group 2 and group3 were significantly lower than group 1.

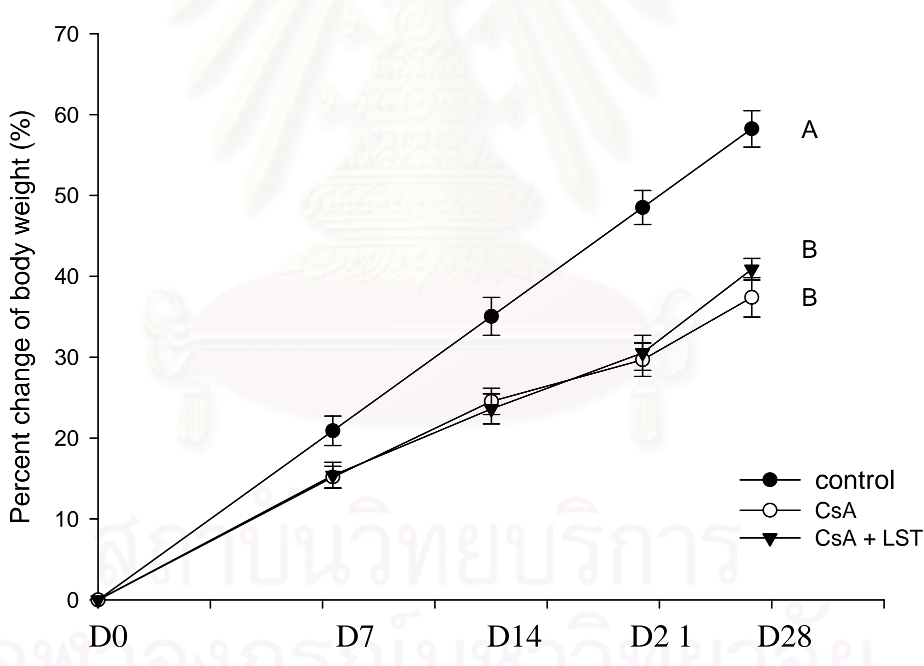

Figure 4.1 Percent changes of body weight at days 0, 7, 14, 21 and 28 in each group. Data are expressed as mean $\pm$ S.E.

^{A,B} Means with different superscripts differ significantly (p <0.05), by using one way ANOVA on rank.

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จุฬาลงกรณ์มหาวิทยาลัย

Feed intake

Feed intake are presented in figure 4.2. There were no significant changes in feed intake in all groups. Mean feed intake were similar before treatment. The average feed intake of group 1, group 2 and group 3 were 21.17 ± 1.52, 24.2 ± 1.48 and 20.86 ± 1.32 g per day, respectively. At day 27 of treatment, the average feed intake of group 1, group 2 and group 3 were 18.36 ± 1.24, 20.99 ± 1.27 and 21.9 ± 1.21 g per day, respectively. There were no significant changes in feed intake in all groups.

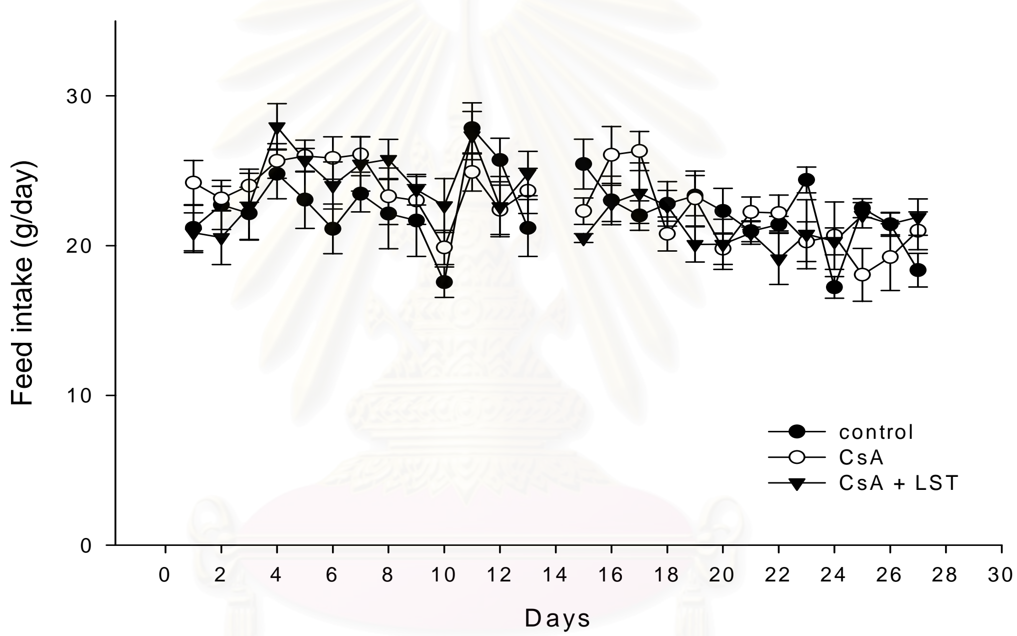

Figure 4.2 Daily mean values for feed intake in three groups of rats throughout the experimental period.

Plasma creatinine and plasma urea nitrogen concentrations

Plasma creatinine and plasma urea nitrogen (PUN) at day 0, day 14 and day 28 in each group are presented in table 4.1, figure 4.3 and figure 4.4. Plasma creatinine and PUN concentrations were not differed at the beginning of the experiment (day 0). At day 28, creatinine concentration was significantly higher than day 0 in all groups when comparing among groups, only group 3 had lower creatinine at day 14 from group 2. The tendency was the same on day 28. The PUN

was significantly increased at day 28 compared to day 0 in all groups, when comparing among groups, significant increases in PUN were found in group 2 which received CsA both on day 14 and 28 ($p<0.05$). In group 3, PUN had tendency to be lower than group 2 although it was slightly higher than control group 1.

Table 4.1 Plasma creatinine and plasma urea nitrogen at day 0, day 14 and day 28 in each group.

	Control (n=21)	**CSA (n=28)**	**CSA + LST (n=21)**
Plasma creatinine (mg%)			
day 0	0.41 ± 0.03^{X}	0.35 ± 0.01^{X}	0.37 ± 0.01^{X}
day 14	$0.40 \pm 0.01^{AB,X}$	$0.44 \pm 0.02^{A,Y}$	$0.37 \pm 0.02^{B,X}$
day 28	0.47 ± 0.02^{Y}	0.52 ± 0.02^{Z}	0.48 ± 0.02^{Y}
Plasma urea nitrogen (mg%)			
day 0	14.70 ± 0.73^{x}	13.52 ± 0.53^{x}	14.11 ± 0.53^{x}
day 14	$15.53 \pm 0.44^{A,xy}$	$27.88 \pm 1.80^{B,y}$	$19.26 \pm 1.22^{A,x}$
day 28	$19.76 \pm 1.08^{A,y}$	$35.98 \pm 3.05^{B,z}$	$26.72 \pm 1.52^{B,y}$

All value are expressed as mean $\pm$ SE

^{A,B} Means in the same row with different superscripts differ significantly ($p<0.05$) using one way ANOVA on rank

^{X,Y,Z} Means in the same column with different superscripts differ significantly ($p<0.05$) using one-way ANOVA with repeated measurement on rank.

^{x,y,z} Means in the same column with different superscripts differ significantly ($p<0.05$) using one-way ANOVA with repeated measurement.

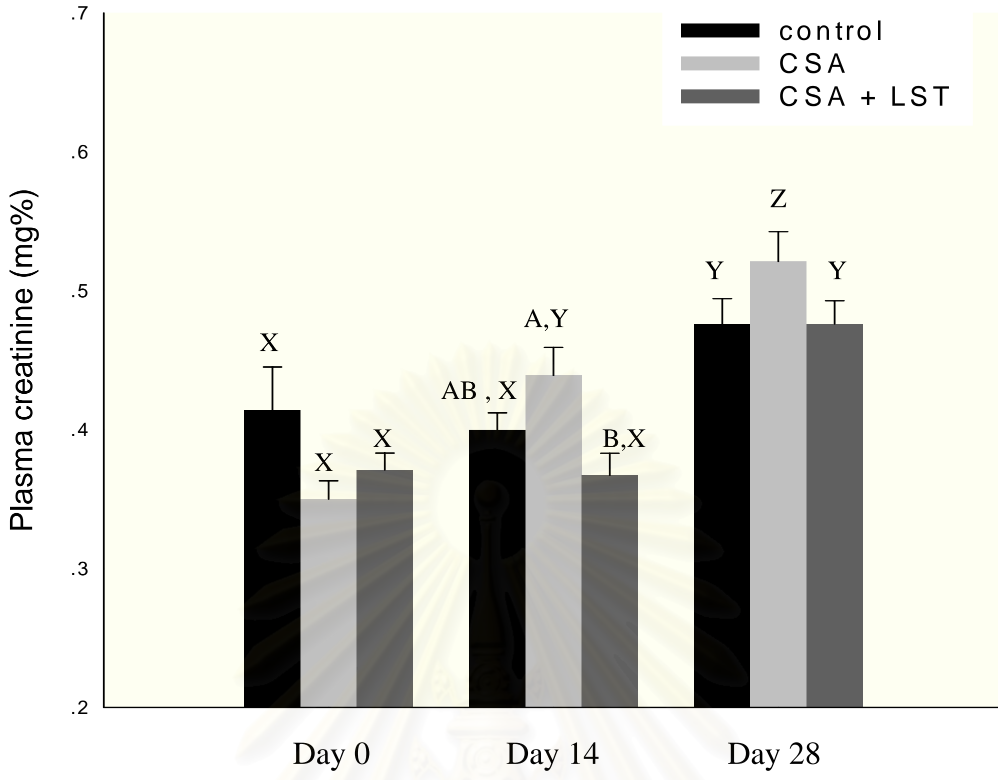

Figure 4.3 Mean values for plasma creatinine concentrations at day 0, day 14 and day 28 in each group. Each bar is expressed as mean $\pm$ SE.

[A,B] Means in the same day with different superscripts differ significantly ($p < 0.05$) using one way ANOVA on rank.

[X,Y,Z] Means in the same group with different superscripts differ significantly ($p < 0.05$) using one-way ANOVA with repeated measurement on rank.

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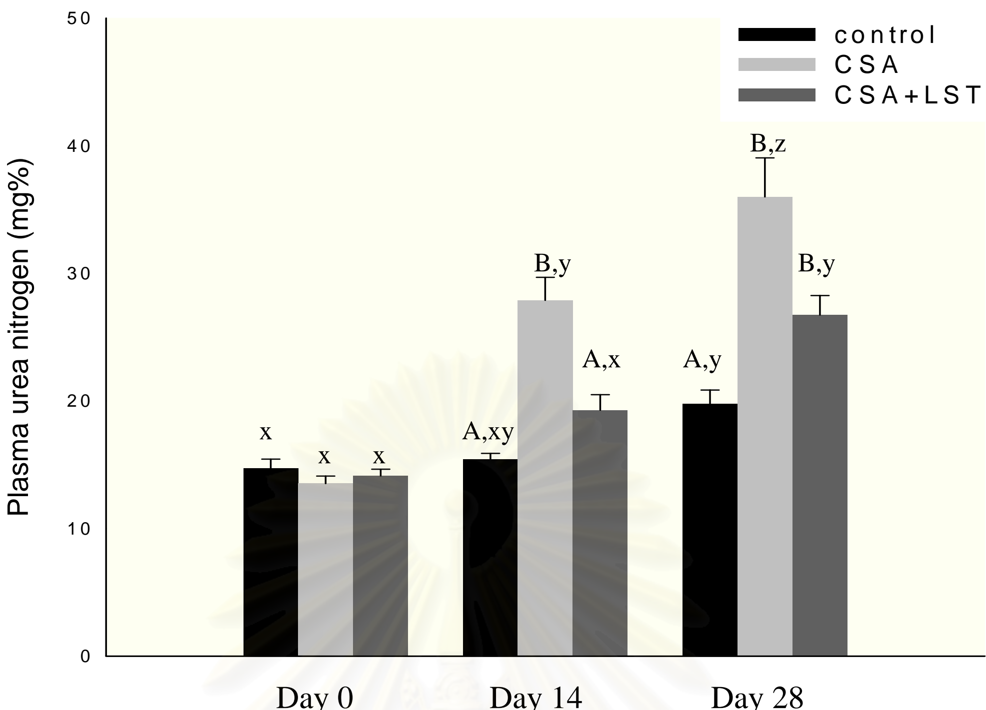

Figure 4.4 Mean values for plasma urea nitrogen at day 0, day 14 and day 28 in each group. Each bar is expressed as mean ± SE.

[A,B] Means in the same day with different superscripts differ significantly ($p < 0.05$) using one way ANOVA on rank.

[x, y, z] Means in the same group with different superscripts differ significantly ($p < 0.05$) using one-way ANOVA with repeated measurement.

Effects of cyclosporine and losartan on renal hemodynamics

Effects of cyclosporine and losartan on glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), filtration fraction (FF) and renal vascular resistance (RVR)

Glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), filtration fraction (FF), renal vascular resistance (RVR) and PCV in each group of rats are shown in table 4.2 and figure 4.5. Glomerular filtration rate decreased significantly in CsA-treated rats. Group 3 which recieved CsA and losartan, GFR was reversed, but it was not different from both

control and CsA treated group. The ERPF was not significantly different among groups. The ERBF was decreased in CsA treated group. Losartan significantly increased effective renal blood flow compared with those receiving CsA alone.

Cyclosporine treatment produced a significant decrease in the FF as compared with control group. Co-treatment with losartan did not affect the FF. The RVR of cyclosporine treated rats appeared to be higher than control group. Concomitant treatment of rats with losartan significantly decreased the RVR as compared to cyclosporine treated group, however it was not different from control. Pack cell volume of cyclosporine treated rats were lower significantly from control rats and losartan treated rats.

Table 4.2 Glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR), filtration fraction (FF) and packed cell volume (PCV) in all group.

	Control (n=9)	**CSA (n=14)**	**CSA + LST (n=9)**
GFR (µl/g/min)	3.89 ± 0.16^{a}	2.88 ± 0.23^{b}	3.31 ± 0.29^{ab}
ERPF (µl/g/min)	18.69 ± 1.74	17.46 ± 1.46	21.92 ± 1.59
ERBF (µl/g/min)	32.24 ± 3.03^{ab}	28.43 ± 2.42^{b}	39.13 ± 3.21^{a}
FF (%)	22.70 ± 2.22^{a}	17.14 ± 1.03^{b}	15.41 ± 1.34^{b}
RVR (mmHg/µl.g^{-1}.min^{-1})	3.76 ± 0.50^{AB}	5.11 ± 0.78^{A}	2.80 ± 0.36^{B}
PCV (%)	41.91 ± 0.96^{A}	38.43 ± 0.32^{B}	43.04 ± 1.55^{A}

All value are expressed as mean $\pm$ SE

^{a,b} Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA.

^{A,B} Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA on rank.

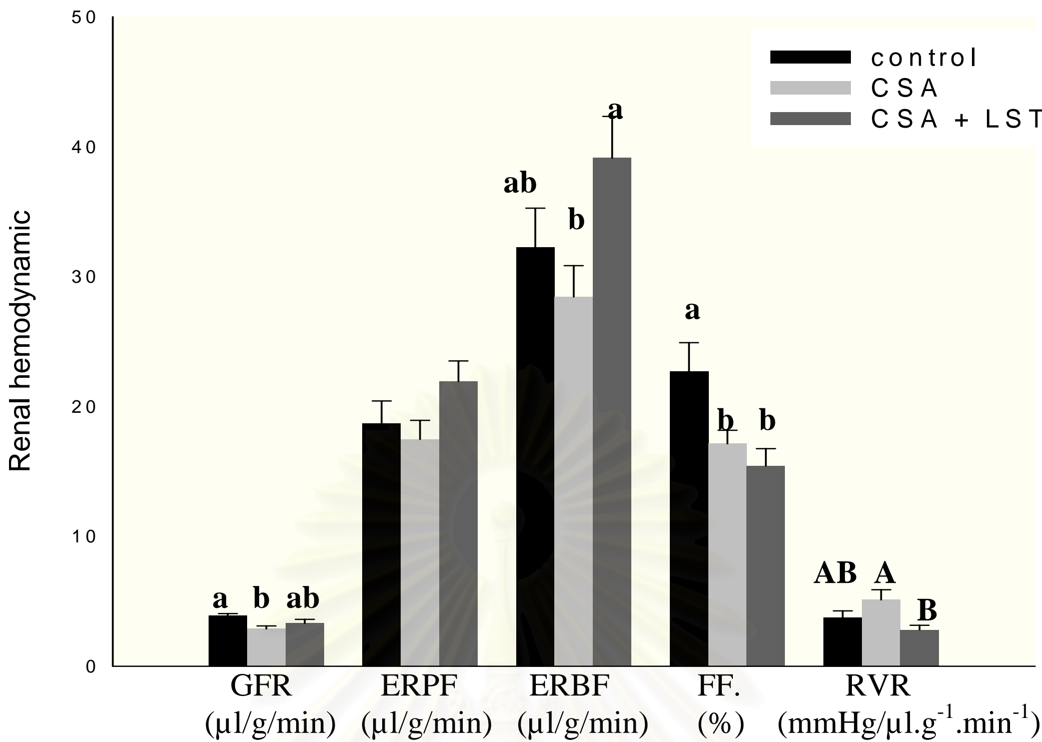

Figure 4.5 Mean values of glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR) and filtration fraction (FF) in all group. The data were shown as mean ± SE.

[a,b] Means with different superscripts differ significantly ($p < 0.05$) using one way ANOVA.

[A,B] Means with different superscripts differ significantly ($p < 0.05$) using one way ANOVA on rank.

Effects of cyclosporine and losartan on mean arterial pressure, and urine flow rate

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Mean arterial pressure (MAP) and urine flow rate at day 28 of treatment in each group are presented in table 4.3. As shown in figure 4.6, MAP was significantly increased in cyclosporine treated rats compared with control rats. Co-treatment with losartan can significantly decrease MAP compared with cyclosporine treated rats and MAP was reversed nearly to control rats. There was no significant changes in urine flow rate in all group.

Table 4.3 Mean arterial pressure (MAP) and urine flow rate at day 28 of treatment in each group

	Control (n=9)	CSA (n=14)	CSA + LST (n=9)
MAP (mmHg)	104.41 ± 3.62^{a}	115.57 ± 2.68^{b}	97.75 ± 4.93^{a}
Urine flow rate (ml/min)	0.012 ± 0.005	0.010 ± 0.001	0.008 ± 0.001

All value are expressed as mean $\pm$ SE

[a,b] Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA.

[A, B] Means in the same row with different superscripts differ significantly ($p < 0.05$) by using one way ANOVA on rank.

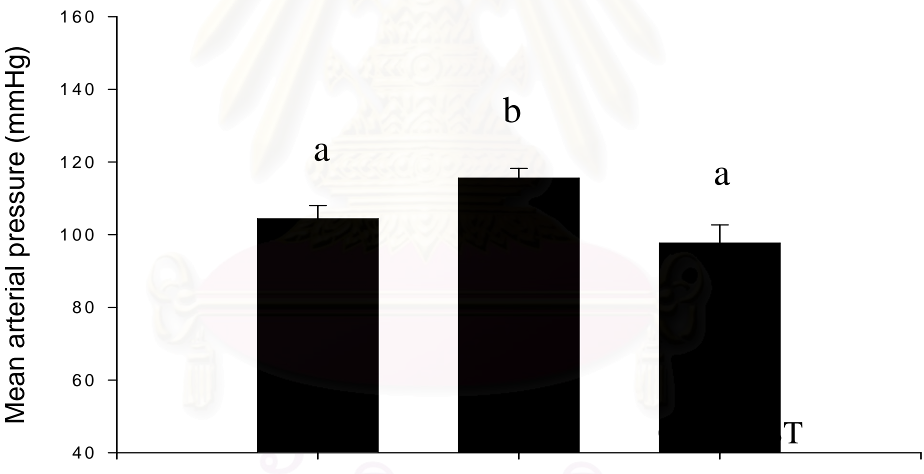

Figure 4.6 Effect of CsA and cotreatment with losartan (LST) on mean arterial blood pressure. The data were shown as mean $\pm$ SE.

[a,b] Means with different superscripts differ significantly ($p < 0.05$) by using one way ANOVA.

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Effects of cyclosporine and losartan on plasma osmolality and plasma electrolytes concentration

The plasma osmolality (Posm) and plasma electrolytes concentrations (Na^+, K^+, Cl^-) are presented in table 4.4. There was significantly increased in plasma osmolality in cyclosporine group as compared to control group. Cyclosporine treatment caused a significant increased in plasma sodium and potassium concentrations compared with those in normal control rats. Plasma chloride concentration was not different between groups. Treatment of CsA in combination with losartan caused similar results compared with rats receiving CsA alone.

Table 4.4 The plasma osmolality (Posm), and plasma electrolyte concentrations in all groups at day 28 of treatment.

	Control (n = 21)	**CSA (n= 28)**	**CSA + LST (n = 21)**
Posm (mosm/l)	311.5 ± 1.4 [a]	323.1 ± 2.6 [b]	316 6 ± 3.6 [ab]
P*Na* (mEq/l)	133.9 ± 1.2 [a]	140.6 ± 1.0 [b]	139.2 ± 2.6 [ab]
P*K* (mEq/l)	4.32 ± 0.15 [a]	4.86 ± 0.15 [b]	5.02 ± 0.17 [b]
P*Cl* (mEq/l)	102.3 ± 1.0	102.5 ± 0.8	101.8 ± 1.1

All value are expressed as mean ± SE.

[a,b] Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA.

Effects of cyclosporine and losartan on osmolar clearance, free water clearance and fractional excretion of electrolytes

No difference was found in Cosm and CH_2O in all groups (table 4.5). The FEK and FECl were not significantly different among groups (table 4.5). In cyclosporine treated rats, FENa was significantly decreased compared to control rats but there was not different from co-treated with losartan (table 4.5).

Table 4.5 Osmolality clearance (Cosm), free water clearance (CH_2O) and fractional excretions (Na^+, K^+, Cl^-) at day 28 in each group.

	Control (n=9)	**CSA (n=14)**	**CSA + LST (n=9)**
Cosm (ml/day)	44.04 $\pm$ 2.22	38.02 $\pm$ 1.61	36.95 $\pm$ 2.09
CH_2O (ml/day)	-26.85 $\pm$ 2.22	-23.74 $\pm$ 1.43	-24.98 $\pm$ 1.84
FE Na (%)	0.347 $\pm$ 0.035[a]	0.275 $\pm$ 0.052 [b]	0.281 $\pm$ 0.030[ab]
FE K (%)	13.29 $\pm$ 1.21	16.25 $\pm$ 2.77	14.621 $\pm$ 2.07
FE Cl (%)	0.256 $\pm$ 0.031	0.251 $\pm$ 0.061	0.228 $\pm$ 0.023

All value are expressed as mean $\pm$ SE.

[a,b] Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA.

Effects of cyclosporine and losartan on urinary protein excretion and urinary protein creatinine ratio

Urinary protein excretion and urinary protein creatinine ratio (UPC ratio) at day 0, 14 and 28 of treatment in each group of rats are presented in table 4.6 and figure 4.7. Urinary protein excretion was elevated from day 0 to 28 in group 1 and 2. At day 28, urinary protein excretion in CsA treated rats were similar to control rats. Concomitant treatment with losartan significantly decreased urinary protein excretion compared with control and cyclosporine treated rats.

Although UPC ratio was not elevated along with the time of treatment, the tendency of reduced UPC ratio after losartan treatment was found. At day 28, UPC ratio remained unchanged in group 2 but was significantly decreased in losartan co-treated rats.

Table 4.6 Urinary protein excretion (mg / day) and urinary protein creatinine ratio (UPC ratio) at 0, 14 and 28 days of treatment in three groups.

	Control (n = 17)	**CSA (n = 24)**	**CSA + LST (n = 18)**
Urinary protein excretion			
day 0	9.60 ± 1.51 ^{X}	11.44 ± 0.89 ^{X}	10.05 ± 0.87
day 14	15.97 ± 1.73 ^{Y}	17.99 ± 1.62 ^{Y}	17.06 ± 2.44
day 28	20.22 ± 1.47 $^{A, Z}$	24.98 ± 8.47 $^{A, Y}$	11.67 ± 1.99 ^{B}
UPC ratio			
day 0	1.92 ± 0.26	1.84 ± 0.13	1.51 ± 0.10
day 14	2.19 ± 0.17	2.09 ± 0.19	2.04 ± 0.25
day 28	2.15 ± 0.11 ^{A}	2.07 ± 0.19 ^{A}	1.36 ± 0.19 ^{B}

All value are expressed as mean ± SE.

$^{A, B}$ Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA on rank.

$^{X, Y, Z}$ Means in the same column with different superscripts differ significantly ($p < 0.05$) using one-way ANOVA with repeated measurement on rank.

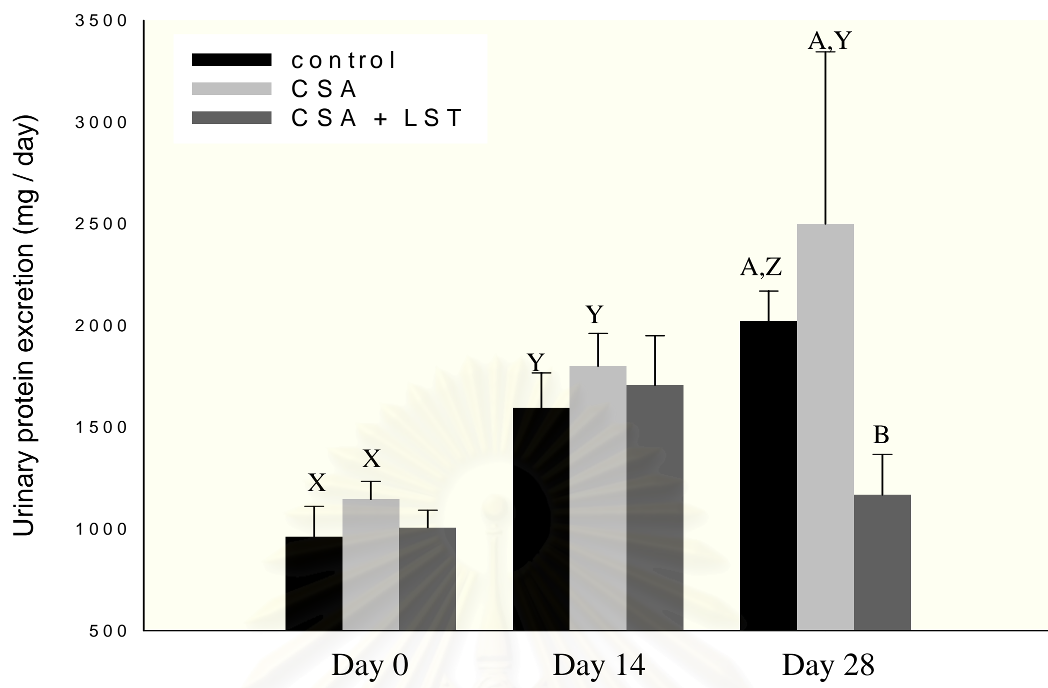

Figure 4.7 Mean values of urinary protein excretion at 0, 14 and 28 days of treatment in three groups. Each bar was shown as mean ± SE.

A,B Means in the same day with different superscripts differ significantly ($p < 0.05$) using one way ANOVA on rank.

X, Y, Z Means in the same group with different superscripts differ significantly ($p < 0.05$) using one-way ANOVA with repeated measurement on rank.

Effects of cyclosporine and losartan on urinary electrolyte excretion

Urinary excretion of the electrolytes (sodium, potassium and chloride) in each group at day 0, day 14 and day 28 are presented in table 4.7. At day 0 and day 14, urinary electrolytes excretion (Na^+, K^+, Cl^-) were not significantly different among groups. Urinary sodium excretion at day 28 of treatment was significantly decreased in cyclosporine treated rats compared with control and losartan group. There was no significant changes in urinary potassium and chloride excretions in all groups.

Table 4.7 Urinary excretion of the electrolytes (sodium, potassium and chloride) in each group at day 0, day 14 and day 28.

Day	**Control (µEq /day)**			**CSA (µEq /day)**			**CSA + LST (µEq /day)**		
	UNa*V	**UK*V**	**UCl*V**	**UNa*V**	**UK*V**	**UCl*V**	**UNa*V**	**UK*V**	**UCl*V**
O	767 ± 57	899 ± 60	424 ± 72	710 ± 47^{X}	811 ± 68	564 ± 50	663 ± 52	864 ± 68	541 ± 62
14	758 ± 55	981 ± 88	569 ± 48	620 ± 38^{XY}	854 ± 53	390 ± 43	646 ± 45	991 ± 64	433 ± 45
28	909 ± 88^{A}	1,145 ± 128	498 ± 47	625 ± 82^{B,Y}	1,146 ± 78	362 ± 47	708 ± 90^{AB}	1,185 ± 116	417 ± 45

All value are expressed as mean ± SE**;** n = 21, 28 and 21, respectively

^{A,B} Means in the same day with different superscripts differ significantly (p <0.05) using one-way ANOVA on rank.

^{X,Y} Means in the same group with different superscripts differ significantly (p <0.05) using one-way ANOVA with repeated measurement on rank.

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Effects of losartan on CsA-induced oxidative stress

Effects of cyclosporine and losartan on urinary malondialdehyde (MDA) excretion

MDA excretion in urine at day 0, 14 and 28 after treatment in each group of rat are presented in table 4.8 and figure 4.8. Urinary MDA excretion of all group tended to be higher from day 0 to day 28. Moreover, by comparing among groups, no significant difference was found in every period of experiment. However, the urinary MDA in rats received cyclosporine plus losartan were likely to be low, compared to other two groups.

Table 4.8 Urinary malondialdehyde excretion (nmol/day) at day 0, 14 and 28 after treatment

	Control (n = 11)	**CSA (n = 14)**	**CSA + LST (n = 12)**
Day 0	109.49 ± 12.05^{x}	117.85 ± 11.65	125.69 ± 13.86
Day14	134.57 ± 4.75^{x}	161.20 ± 14.28	145.24 ± 10.97
Day 28	175.33 ± 13.31^{y}	182.81 ± 25.10	152.20 ± 13.44

The data were shown as mean ± SE

^{x,y} Means in the same column with different superscripts differ significantly ($p < 0.05$) using one-way ANOVA with repeated measurement.

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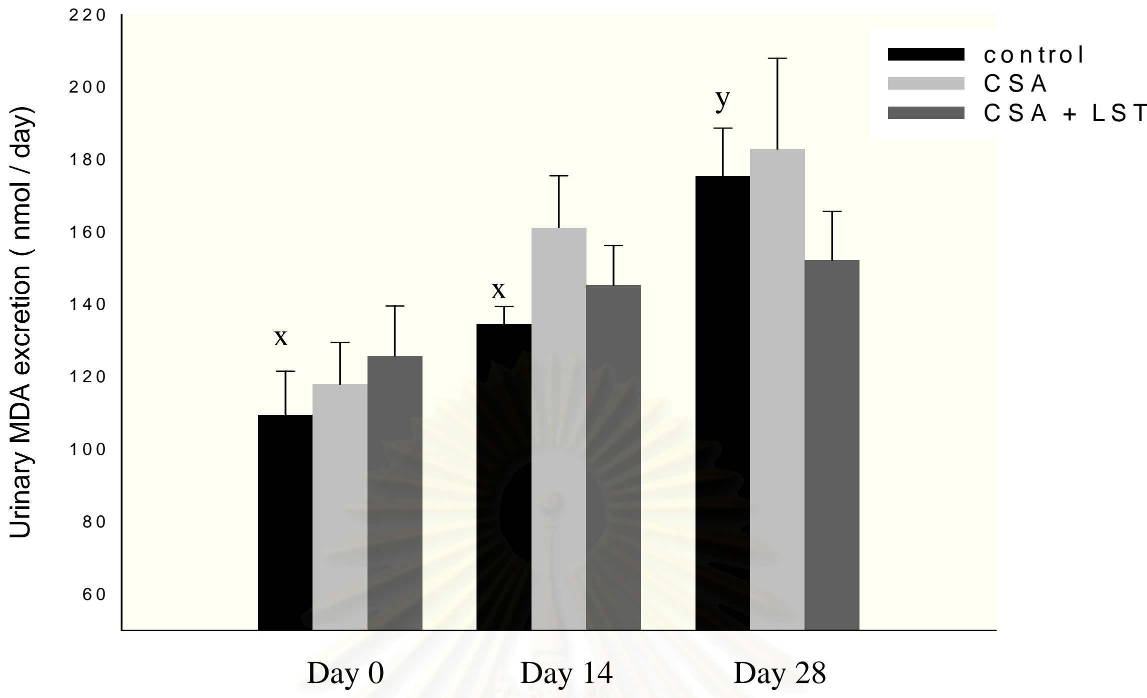

Figure 4.8 Mean values of urinary MDA excretion at day 0, 14 and 28. The data are expressed as mean ± SE.

Effects of cyclosporine and losartan on urine MDA creatinine ratio (nmol / mg creatinine)

There was no significant change in the urine MDA creatinine ratio in any treatment group (table 4.9 , figure 4.9)

Table 4.10 Urinary malondialdehyde creatinine ratio (nmol/ mg creatinine) at day 0, 14 and 28 after treatment.

	Control (n=11)	CSA (n=14)	CSA + LST (n=12)
Day 0	16.76 ± 2.22	17.43 ± 2.51	20.75 ± 1.97
Day14	20.46 ± 2.66	17.58 ± 1.72	17.66 ± 1.61
Day 28	17.82 ± 2.02	21.02 ± 4.14	20.46 ± 2.88

The data are expressed as mean ± SE.

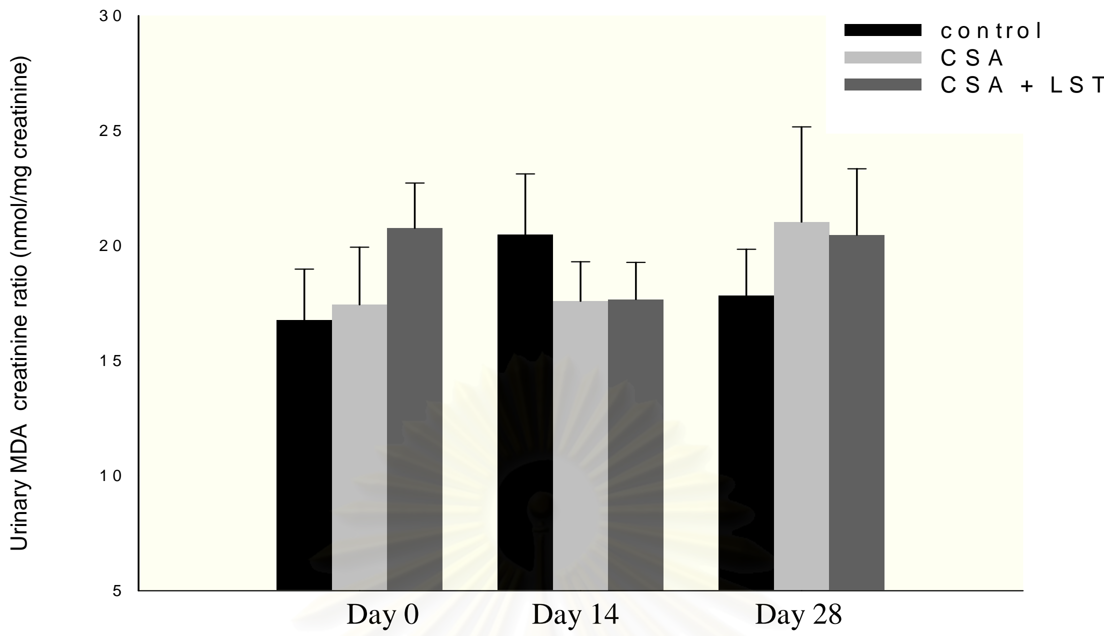

Figure 4.9 Mean values of urinary MDA creatinine ratio at day 0, 14 and 28
The data are expressed as mean ± SE.

Effect of cyclosporine and losartan on kidney reduced glutathione (GSH), malondialdehyde (MDA), catalase (CAT) and plasma MDA

Cyclosporine produced a significant reduction in renal reduced glutathione concentrations compared with control group (table 4.10 and figure 4.10). As shown in table 4.10 and figure 4.11, there was no significantly different of renal catalase activity between groups. The kidney MDA and plasma MDA concentrations were significantly increased in cyclosporine treated rats compared with control rat (table 4.10, figure 4.12 and figure 4.13). Co-treatment with losartan inhibited cyclosporine induced lipid peroxidation and resulted in a significant decrease in MDA level in kidney. There was a decrease in plasma MDA concentration in the losartan co-treated group; although no differ significant compared with CsA treated group.

Table 4.10 Effect of cyclosporine and losartan on kidney reduced glutathione, catalase activity, kidney and plasma malondialdehyde.

	Control (n = 12)	CSA (n = 14)	CSA+LST (n = 12)
Kidney			
GSH (nmol / mg protein)	33.09 ± 0.86^{a}	29.84 ± 0.84^{b}	33.16 ± 1.43^{ab}
Catalase (U / mg protein)	49.50 ± 3.44	47.91 ± 1.98	55.16 ± 1.98
MDA (nmol / mg protein)	1.21 ± 0.04^{a}	1.44 ± 0.06^{b}	1.20 ± 0.05^{a}
Plasma			
MDA (nmol /ml)	4.87 ± 0.37^{a}	6.67 ± 0.37^{b}	5.13 ± 0.21^{ab}

The data are expressed as mean ± SE.

[a,b] Means in the same row with different superscripts differ significantly ($p < 0.05$) using one way ANOVA.

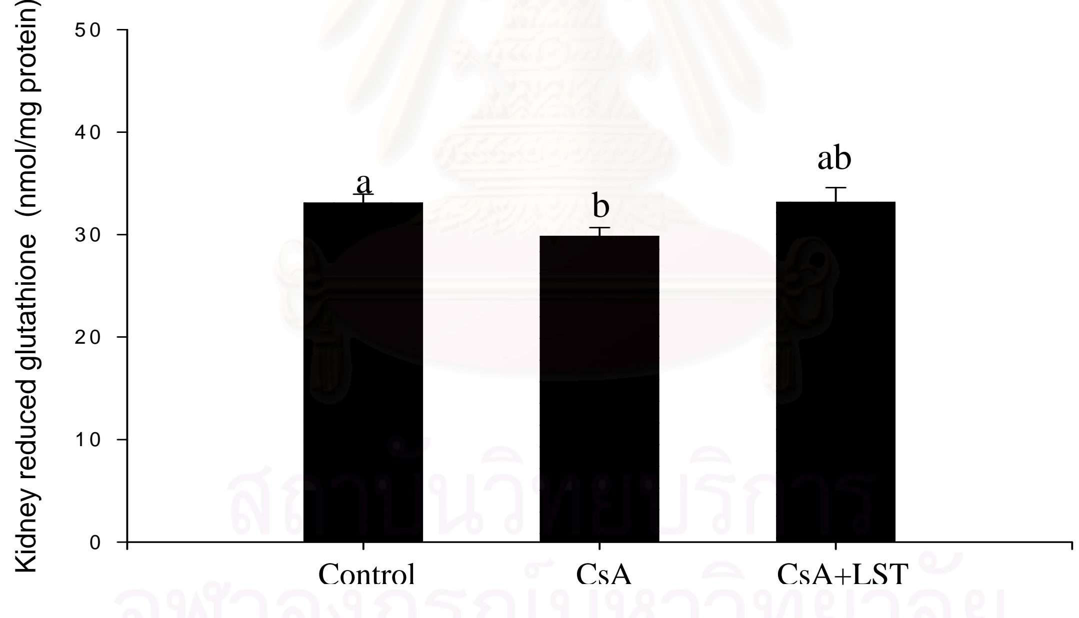

Figure 4.10 Effect of cyclosporine (CsA) and losartan (LST) on kidney reduced glutathione. The data are expressed as mean ± SE. [a,b] Means with different superscripts differ significantly ($p < 0.05$) by using one way ANOVA.

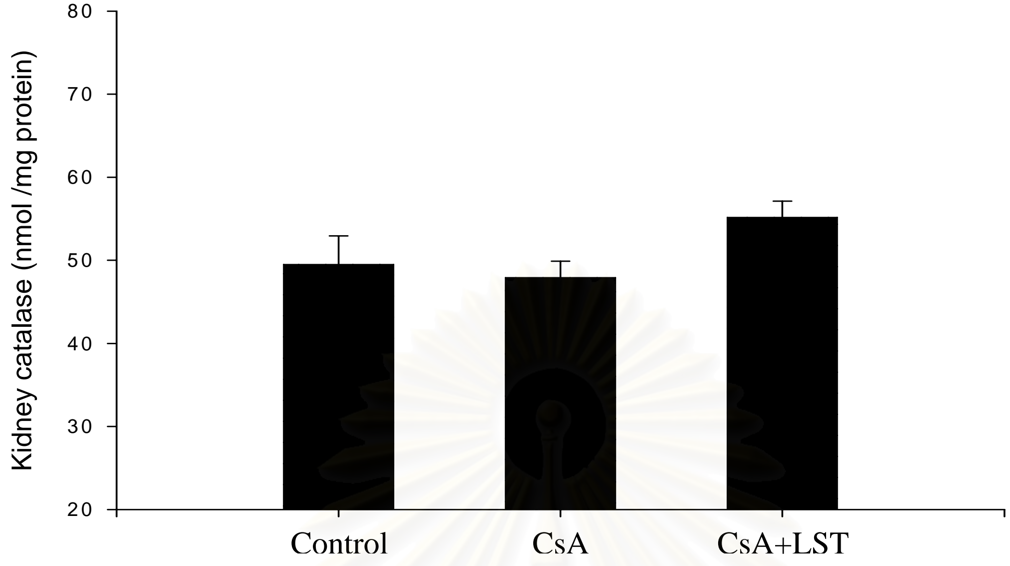

Figure 4.11 Effect of cyclosporine (CsA) and losartan (LST) on kidney catalase The data are expressed as mean + SE.

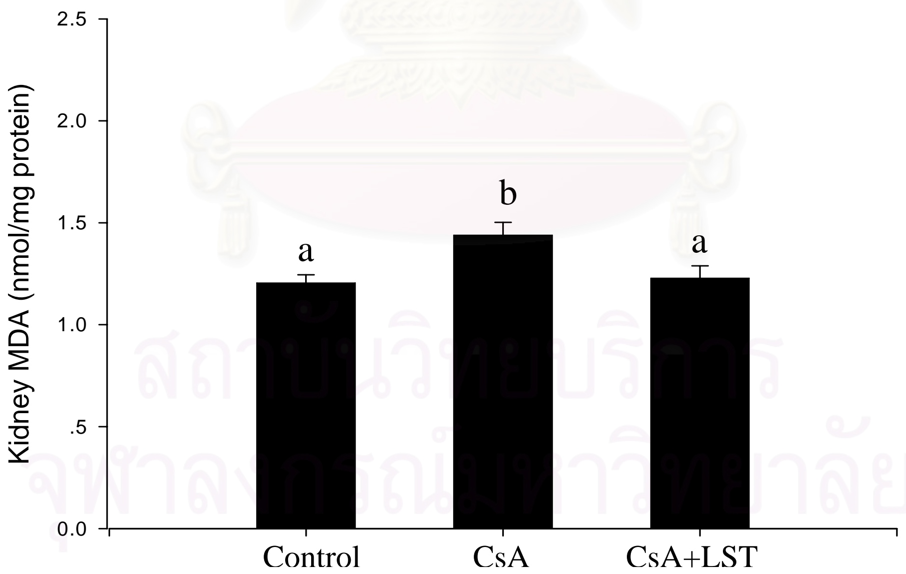

Figure 4.12 Effect of cyclosporine (CsA) and losartan (LST) on kidney MDA concentration. The data are expressed as mean + SE.

[a,b] means with different superscripts differ significantly ($p < 0.05$) by using one way ANOVA.

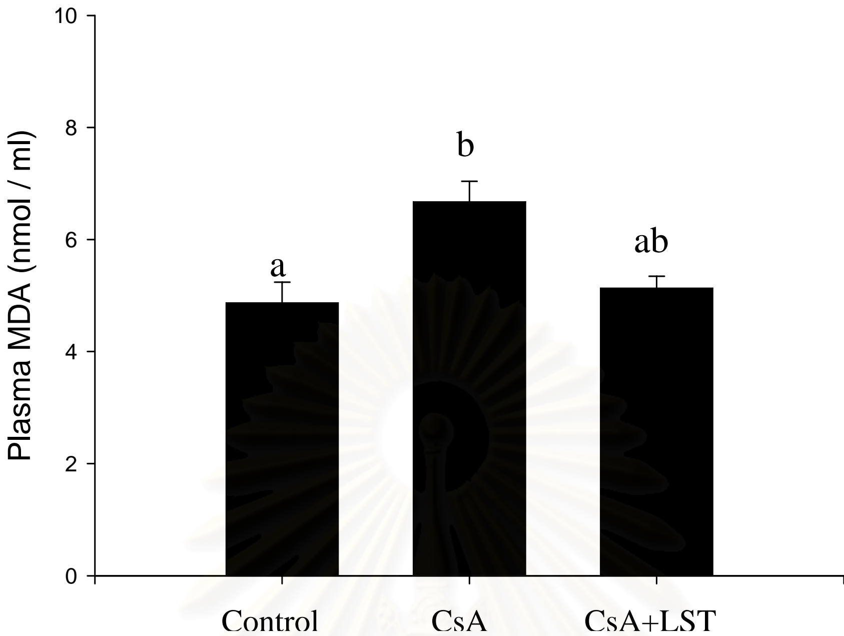

Figure 4.13 Effect of cyclosporine (CsA) and losartan (LST) on plasma MDA concentration. Each bar represents the mean ± SE.

[a,b] means with different superscripts differ significantly ($p < 0.05$) by using one way ANOVA.

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Effects of cyclosporine and losartan on kidney and plasma catecholamine contents

There were increases in plasma norepinephrine, epinephrine and dopamine contents in cyclosporine treated rats but not significant different from control group. Losartan co-administration decreased plasma norepinephrine and epinephrine compared with cyclosporine treated group but not statistically significant. The plasma dopamine concentration was increased markedly in cyclosporine cotreatment with losartan group (table 4.11 and figure 4.14).

Cyclosporine treatment caused significant increases in kidney norepinephrine and epinephrine contents as compared to control rats. Concomitant treatment with losartan significantly prevented an increase in the kidney norepinephrine contents. (table 4.11 and figure 4.15). Kidney dopamine concentrations was slightly higher in CsA group and declined in group 3 which received losartan. The chromatograms represent NE, E and DA levels in plasma and kidney in each group were shown as figure 4.16 and 4.17, respectively.

Table 4.11 Plasma and kidney concentrations of norepiephrine, epinephrine and dopamine in each group.

	Control (n=12)	CSA (n=14)	CSA+LST (n=12)
Plasma (ng / ml)			
Norepinephrine	10.11 ± 1.03	15.32 ± 2.51	13.99 ± 2.10
Epinephrine	19.18 ± 1.88	32.56 ± 6.42	22.83 ± 3.47
Dopamine	3.88 ± 0.57	4.51 ±0.78	5.64 ± 0.73
Kidney (ng / mg protein)			
Norepinephrine	2.20 ± 0.16[a]	2.82 ± 0.13[b]	2.27 ± 0.15[a]
Epinephrine	0.28 ± 0.04[a]	0.45 ± 0.06[b]	0.34 ± 0.31[ab]
Dopamine	0.29 ± 0.03	0.35 ± 0.05	0.27 ± 0.02

The data are expressed as mean ± SE.

[a,b] Means in the same row with different superscripts differ significantly ($p < 0.05$) by used one way ANOVA.

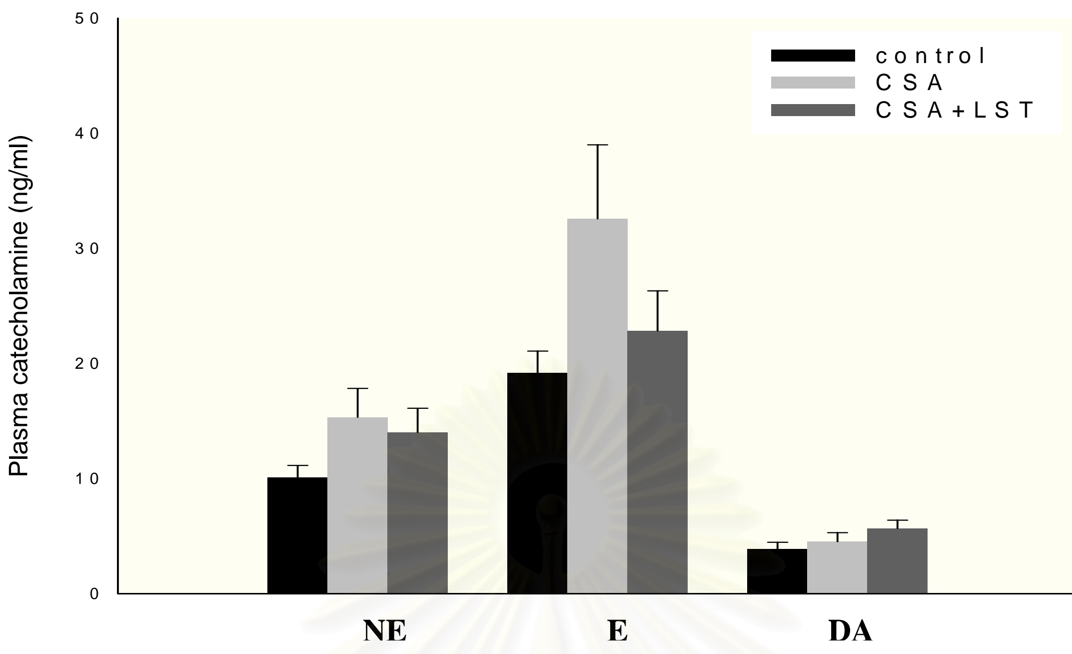


Figure 4.14 Plasma concentrations of norepinephrine, epinephrine and dopamine in each group. Each bar represents the mean ± SE.

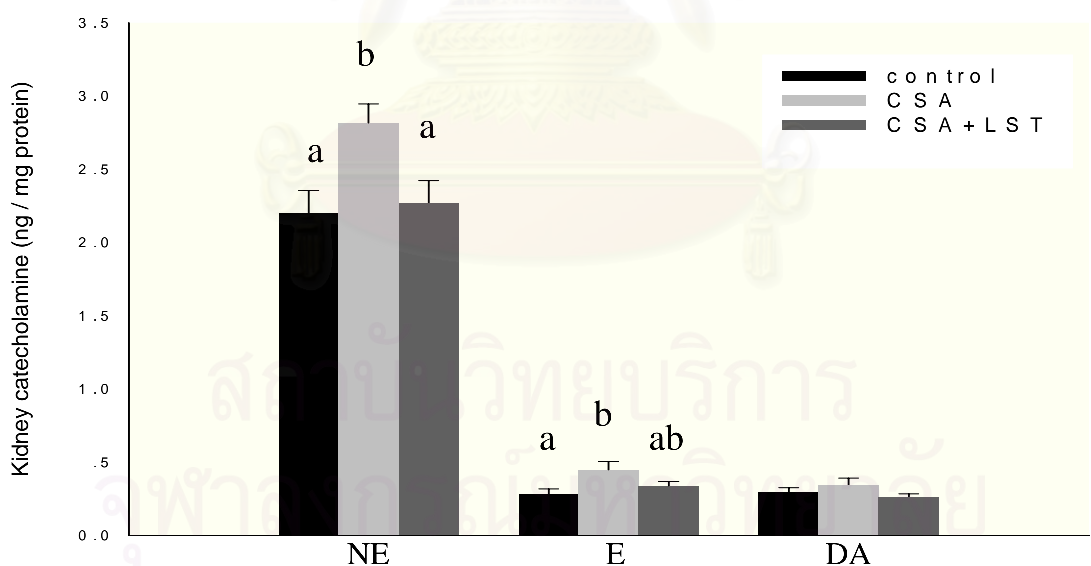


Figure 4.15 kidney concentrations of norepinephrine, epinephrine and dopamine in each group. Each bar represents the mean ± SE.

[a ,b] indicates the difference ($p < 0.05$) between the control group, cyclosporine treated group and cyclosporine cotreatment with losartan group by used one way ANOVA.

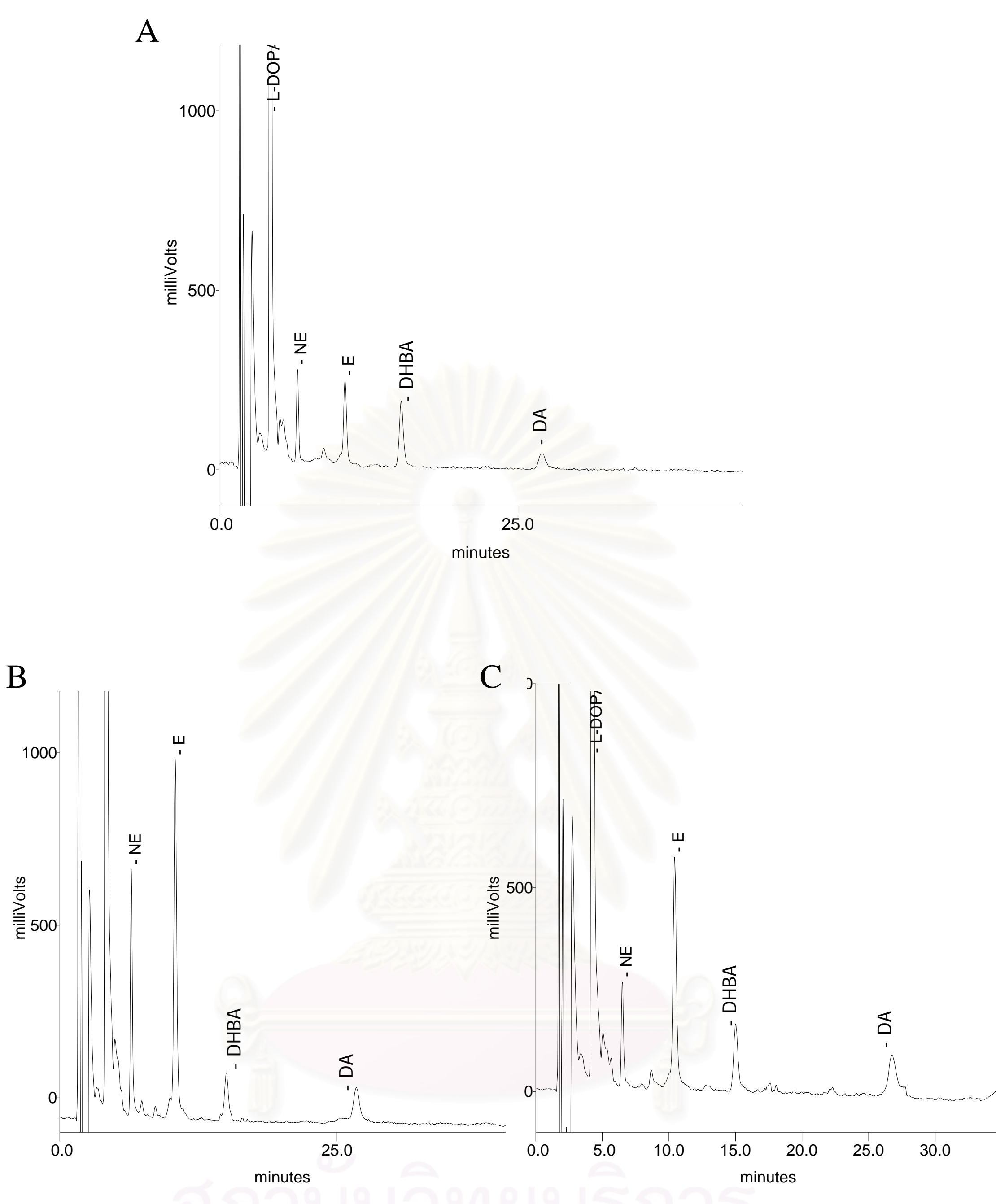
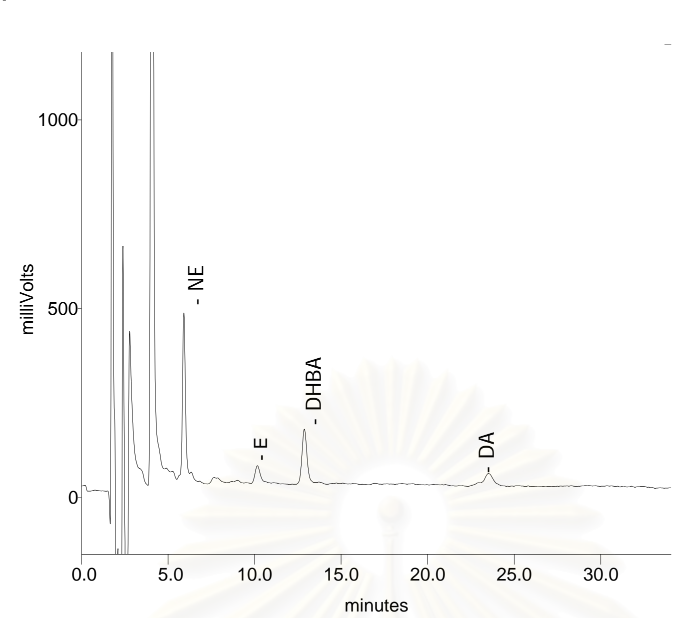


Figure 4.16 The chromatograms represent NE, E and DA levels in plasma of (A) control (CON), (B) CsA, (C) CsA + LST rats measured by HPLC-EC.

A



B C

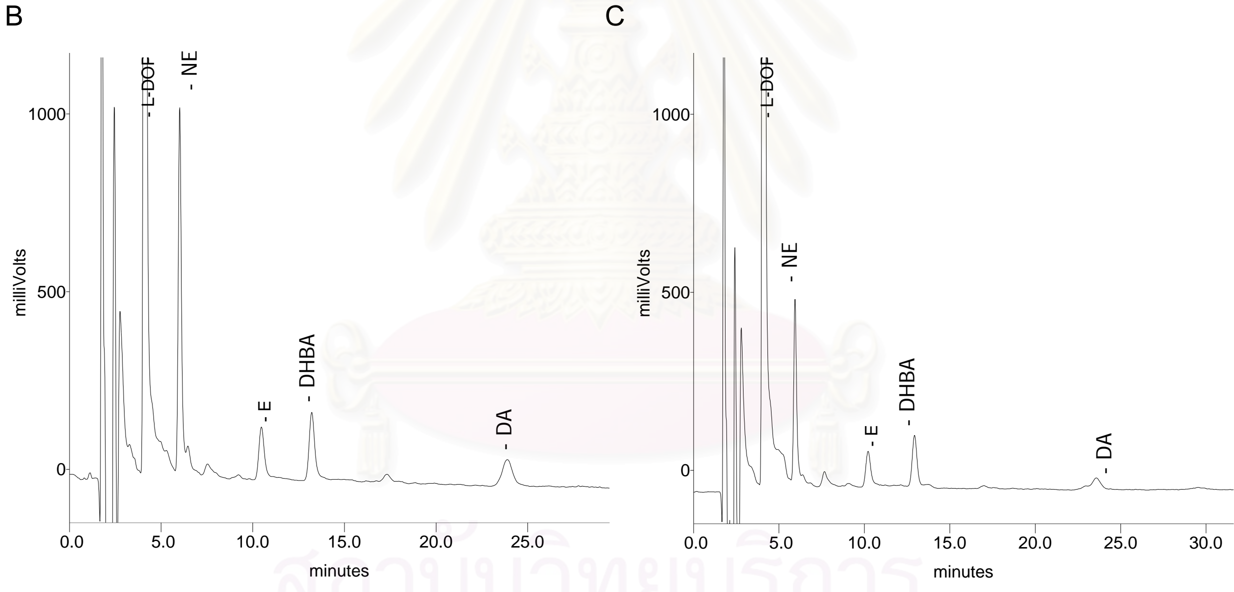


Figure 4.17 The chromatograms represent NE, E and DA levels in kidney of (A) control (CON), (B) CsA, (C) CsA + LST rats measured by HPLC-EC.

CHAPTER V

DISCUSSIONS

In the present study, CsA significantly reduced body weight gain with no effect on feed intake. This is in agreement with the study of Shaltout and Abdel-Rahman (2003), they suggested that CsA caused weight loss may be due to increased catabolism. However, the reasons for the reduction in body weight in CsA-treated animals was not yet fully understood.

The major adverse effects of CsA immunosuppression are nephrotoxicity and hypertension (Mason., 1989; Zhang and Victor, 2000). In the present study, CsA induced renal dysfunction and an increase in blood pressure. These results were consistent to the results study in dog (Ouisuwan and Buranakarl, 2005). Treatment of rats with CsA for a period of 28 days resulted in a significant increase in plasma urea nitrogen, suggesting a functional impairment of kidney. These results are in agreement with earlier investigators, who reported significant alteration in plasma urea nitrogen in patients and experimental animals following treatment with cyclosporine (Mason, 1989). In this study, at day 28, plasma creatinine concentration was higher than day 0 in all groups which may be an age-relate effect. Typically, the decrease in renal function is better characterized by an increase in plasma urea nitrogen rather than plasma creatinine concentration (English et al., 1987; Laskow et al., 1988; Myer et al., 1988). Treatment with losartan decreased both plasma creatinine and plasma urea nitrogen levels in cyclosporine treated rats. The results indicate that CsA-induced glomerular dysfunction was ameliorated by treatment with losartan.

CsA induced nephrotoxicity is characterized by reduced GFR, decreased renal blood flow and increased renal vascular resistance. These effects were found in the present study. Cyclosporine therapy has generally been shown to reduce both glomerular filtration rate and renal plasma flow (Mason, 1989). Murray et al. (1985) found a marked decrease in renal blood flow following both the acute infusion of CsA and 1 week after chronic administration in rats. These changes were seen after CsA infusion which suggests that renal vasoconstriction is a primary effect of CsA.

Myers et al. (1988) found that CsA reduced glomerular filtration rate and renal blood flow by causing vasoconstriction of glomerular afferent arterioles. In the present study, there is a reduction on renal plasma flow which may be due to afferent arteriole vasoconstriction, decreased glomerular capillary pressure which may result in a decrease in GFR. In general, GFR may decrease from low filtration pressure, decreased surface area and/or decreased permeability of the filtering membrane. Previous study reported that GFR and renal plasma flow both decreased while the filtration fraction remained unchange or decreased at high doses in CsA treatment (Mason, 1989).

It has been suggested that CsA-induced hypertension may be due to vasoconstriction of the renal microcirculation, resulting in increased renal vascular resistance with a concomitant decrease in GFR (Devarajan et al., 1989). Besides, Shaltout and Abdel-Rahman (2003) suggested that CsA induced progressive attenuation of baroreceptor sensitivity. Moreover, Ouisuwan and Buranakarl (2005) found that CsA increased blood pressure in normotensive dogs but heart rate was not changed. These findings suggested that baroreceptor reflex may be reset after blood pressure changed for a period of time. Increased blood pressure without heart rate suppression may be due to impaired baroreceptor function (Ryuzaki et al., 1997). CsA reduced the sensitivity of baroreceptor reflex, which coincided with increased pressure setpoint. The effect of CsA on blood pressure and baroreflex may be mediated by AII, since they can be prevented by enalapril, an ACE inhibitor (Ouisuwan and Buranakarl, 2005).

CsA associated hypertension in CsA-treated renal transplant recipients may be linked to CsA-mediated sodium retention (Curtis et al., 1988). Ciresi and coworkers (1992) demonstrated that chronic cyclosporine administration in dogs activated the renin-angiotensin-aldosterone system, suppressed circulating ANF and resulted in active sodium retention. Furthermore, there was an attenuated natriuretic and diuretic response to the acute challenge of intravenous volume expansion in the cyclosporine treated dogs which resulted from enhanced tubular reabsorption of sodium. Possible reasons for enhanced sodium reabsorption include direct renal tubular, vascular and hormonal factors which may alter by CsA administration. In the present study suggested that CsA administration results in sodium retention associated with alteration of sympathetic activity and renin-angiotensin aldosterone system. Sodium

retention was examined by decrease FE_{Na} and $U_{Na}V$ observed in the present study. Decreased FE_{Na} has been suggested to be an early marker of CsA nephrotoxicity after renal transplantation (Morales et al., 1990). Moreover, reduced GFR with a concomitant fall in the fractional excretion of sodium and an expansion of the extracellular fluid volume were found in CsA treated rats (Ciresi et al., 1992).

The urinary protein excretion was not increased in CsA treated group although the previous study was demonstrated (Lassila et al., 2000). However, inhibition of RAS by losartan could reduce urinary protein excretion and UPC ratio as have been demonstrated earliered in patients received CsA (Hausberg et al., 1999). In this study, group 3 had even lower protein excretion more than control group. Whether, decreased protein excretion was indicated through RAA system induced by CsA or the mechanism related to ion selectively of filtration membrane remained unclear.

The mechanism responsible for these effect of CsA has been the subject of much controversy. A possible role for enhanced sensitivity to a variety of vasoconstrictors, including norepinephrine, AII, thromboxane A_2 and endothelin (Cid et al., 2003; Kupferman et al., 1994; Murray et al., 1985) was demonstrated. In the present study, renal norepinephrine concentrations were increased significantly in CsA induced nephrosis rats. When CsA cotreatment with losartan, kidney norepinephrine concentrations were significantly decreased from the CsA alone. These results suggested that activation of renal nerve and intrarenal renin-angiotensin-aldosterone system may play an important role in CsA-induced nephrotoxicity. Several studies indicated that CsA activates sympathetic nerve activity in human and animals (Ryuzaki et al., 1997; Scherrer et al., 1990; Zhong et al., 1998). Previous studies reported cyclosporine increased the norepinephrine release from the sympathetic nervous endings of rat aorta and involves synapsin in renal sensory nerve ending (Tavares et al., 2003; Zhang et al., 2000). Likewise, Scherrer et al. (1990) measured muscle sympathetic nerve traffic in cardiac transplant recipients and patients with myasthenia gravis who were treated with CsA. They found that sympathetic nerve firing was increased, suggesting that CsA induced hypertension was mediated via the sympathetic nervous system. Conversely, Kaye et al. (1993) described the arterial concentrations of norepinephrine and the rate of norepinephrine spillover into plasma in CsA treated cardiac transplant recipients were similar to those of age-matched controls. Furthermore, the renal vasoconstriction associated with CsA therapy was not associated with elevated renal norepinephrine spillover. In addition,

the arterial concentration of ^{3}H-DHPG formed by intraneuronal metabolism of norepinephrine after neuronal uptake was normal. So, these studies were suggested that the disposition of neuronally released norepinephrine is not affected by CsA. In accordance, Sehested et al. (1992) and Van den Dopel et al. (1996) found no change in plasma norepinephrine levels in patients with kidney transplant and hypertension undergoing treatment with cyclosporine. Therefore, central sympathetic nerve activity may not be activated since plasma norepinephrine was unchange. However CsA may activate local sympathetic activity in the kidney.

It is also known that CsA increases renal nerve activity and angiotensin II (Murray et al., 1985). Since renal nerve stimulation increases intrarenal AII formation, it is possible that CsA-induced activation of sympathetic nerve activity is involved in the augmentation of AII production. Sympathetic stimulation may account for the enhanced activity of intrarenal RAS (Dibona, 2004). In this study was supported by reduced in renal norepinephrine contents after losartan treatment. However other factors rather than sympathetic activity and AII may be involved in renal vasoconstriction. Previous studies showed that renal denervation only partially reverses the renal vasoconstriction following acute and chronic CsA treatment (Murray and Paller, 1986; Thomson et al., 1989). Therefore, arterial baroreceptor mechanism may be involved in CsA induced nephrotoxicity.

In this study, the hypertensive effects of CsA was mostly due to the effect of angiotensin II on vasoconstriction since giving CsA in the presence of losartan prevented blood pressure elevation. The renal sympathetic nerves innervate the renin-containing juxtaglomerular granular cells (JG), the tubules and the arterial resistance vasculature (Dibona, 2004). Increases in renal sympathetic nerve activity (RSNA) can increase activity of renin-angiotensin system by stimulating renin release from JG cells. Angiotensin II, through direct actions on AT_1 receptors located on tubular and vascular segments, can also increase renal tubular sodium reabsorption and renal vasoconstriction (Dibona, 2000). Previous study observed that intrarenal generation of AII facilitated renal venous outflow of norepinephrine during renal sympathetic nerve stimulation. This effect was blocked by an AII receptor antagonist. The results suggested that a presynaptic action of angiotensin II on renal nerve terminal may enhance norepinephrine release (Boke and Malik, 1983). Losartan may cause dose-dependently decrease in renal vasoconstrictor response to renal sympathetic nerve stimulation but not to injection of norepinephrine (Dibona, 2000).

CsA increases renin release directly from JG cells with accompanying hyperplasia of the juxtaglomerular apparatus (Kurtz et al., 1988). Moreover, CsA treatment enhances angiotensin converting enzyme activity, upregulates AT_1 receptors in vascular and renal tissue (Avdonin et al., 1999; Tufro-Mcreddie et al., 1993). Avdonin and coworkers (1999) suggested that at least one of the mechanisms by which CsA enhanced blood vessel contractility and blood pressure in humans could be by increasing AT_1 receptors and AII action.

In the present study, CsA treated rats had an increased in plasma and renal lipid peroxides measured as MDA, indicating increased ROS activity. CsA causes vasoconstriction in the kidney (Barros et al., 1987). These alterations could lead to a classical hypoxia-reoxygenation injury involving free radicals. Previous study shown that CsA administration in rats resulted in excess local production of hydroxy radical, leading to lipid peroxidation and nephrotoxicity (Wang and Salahudeen, 1995; Zhong et al., 1998). Result of CsA increased MDA in isolated hepatic and renal microsomes, the major metabolic site for CSA had been demonstrated (Zhong et al., 1998). Therefore, it is possible that metabolism of CsA by cytochrome *P* -450 could directly produced free radicals.

Cyclosporine induced sympathetic nerve activation and ROS production in the kidney were prevented by renal denervation (Zhong et al., 1999). Therefore, it is possible that cyclosporine increases ROS production by increasing renal sympathetic nerve activity resulting in vasoconstriction. Recently, it has been demonstrated that angiotensin II stimulates NAD(P)H oxidase-dependent superoxide production in kidney, proximal tubular cells and vascular smooth muscle cells which is transduced through AT_1 receptors. The superoxide produced on angiotensin II stimulation is rapidly converted to other ROS. In this study, enhancement of CsA-induced nephrotoxicity by glutathione depletion are consistent with other previous investigation (Hager et al., 2006). Futhermore, in the present study losartan attenuates oxidative stress in CsA-treated rats. It is suggest that AII is involved in oxygen free radicals formation and subsequent lipid peroxidation in the kidney (Padi and Chopra, 2002). Whether sympathetic activation was increased ROS formation in this study could not be rule out.

In conclusion, the relationship between AII and renal sympathetic activity was demonstrated and may be involved in pathogenesis of nephrotoxicity and hypertension. Inhibition of RAA system caused reduction in both ROS and renal norepinephrine content. Blocking with AT_1 receptor antagonist can be ameliorate renal dysfunction induced by CsA; However, further study should be done to confirm this mechanism.

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