ผลของการให้วิตามินอีร่วมกับวิตามินซีในการปกป้องการทำหน้าที่ของไต
และระดับของโปรตีนโคลโธ ในหนูแรทที่มีภาวะออกซาเลตในปัสสาวะสูง

นางสาวอรพรรณ จาตุรกาญจน์

จุฬาลงกรณ์มหาวิทยาลัย
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# Protective effects of combined vitamin E and vitamin C supplementation on renal functions and Klotho protein levels in hyperoxaluric rats

Miss Orapun Jaturakan


A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Animal Physiology
Department of Veterinary Physiology
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การศึกษาผลของการให้วิตามินอีร่วมกับวิตามินซีต่อการทำหน้าที่ของไต ภาวะเครียดออกซิเดชัน และระดับของโปรตีนโคลโธ ในหนูแรทที่มีภาวะออกซาเลตในปัสสาวะสูง เป็นระยะเวลา 21 วัน โดยแบ่งการศึกษาเป็น 2 ส่วน ทำการแบ่งโดยการสุ่มหนูทดลองจำนวน 70 ตัวออกเป็น 5 กลุ่ม กลุ่มที่ 1 กลุ่มควบคุม กลุ่มที่ 2 กลุ่มที่เหนี่ยวนำให้มีภาวะออกซาเลตในปัสสาวะสูง กลุ่มที่ 3 กลุ่มที่เหนี่ยวนำให้มีภาวะออกซาเลตในปัสสาวะสูง และได้รับวิตามินอี กลุ่มที่ 4 กลุ่มที่เหนี่ยวนำให้มีภาวะออกซาเลตในปัสสาวะสูง และได้รับวิตามินซี กลุ่มที่ 5 กลุ่มที่เหนี่ยวนำให้มีภาวะออกซาเลตในปัสสาวะสูง และได้รับวิตามินอีร่วมกับวิตามินซี โดยทำการเหนี่ยวนำให้เกิดภาวะออกซาเลตในปัสสาวะสูง ด้วยการผสมสารไฮดรอกซีแอลโพรลีนในน้ำดื่ม ทำการฉีดวิตามินอีเข้าช่องท้องหนูทดลองในขนาด 200 มก./กก. จำนวน 2 ครั้งต่อสัปดาห์ในกลุ่มที่ 3 และ 5 และทำการฉีดวิตามินซีเข้าหลอดเลือดขนาด 500 มก./กก. จำนวน 1 ครั้งต่อสัปดาห์ในกลุ่มที่ 4 และ 5 เมื่อสิ้นสุดการศึกษาทำการศึกษาเคลียรานซ์ในการศึกษาส่วนที่ 1 ในขณะที่การศึกษาในส่วนที่ 2 ทำการฉีดสารสำหรับการดองรักษาชิ้นเนื้อโดยวิธีฉีดน้ำยาเข้าไตผ่านหลอดเลือดแดงใหญ่ในช่องท้องเพื่อทำการเก็บตัวอย่างชิ้นเนื้อไตในวันที่ 21

เมื่อสิ้นสุดการศึกษาพบว่า การขับทิ้งออกซาเลตในปัสสาวะของกลุ่มที่ 2 เพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม ($p<0.05$) ในขณะที่กลุ่มที่ 3 และ 5 มีการขับทิ้งออกซาเลตในปัสสาวะคงที่ อัตราการกรองผ่านกลอเมอรูลัส และการดูดกลับของน้ำและโซเดียมที่ท่อไตส่วนต้นในกลุ่มที่ 2 ลดลงอย่างอย่างมาก และเพิ่มขึ้นในกลุ่มที่ 3, 4 และ 5 นอกจากนี้ พบว่าสถานะรวมของสารต้านอนุมูลอิสระในปัสสาวะของกลุ่มที่ 2 มีระดับลดลง ในขณะที่ระดับมาลอนไดดีไฮด์ในเลือดของกลุ่มที่ 2 มีระดับเพิ่มสูงขึ้น เมื่อเทียบกับกลุ่มที่ 1 และระดับของพารามิเตอร์ทั้งสองจะมีการเปลี่ยนแปลงที่ดีขึ้น เมื่อได้รับสารต้านอนุมูลอิสระเพียงชนิดเดียว หรือร่วมกันทั้งสองชนิด (กลุ่มที่ 3, 4 และ 5) พบปริมาณผลึกของแคลเซียมออกซาเลตในปัสสาวะเพิ่มขึ้นในกลุ่มที่ 2 แต่เมื่อได้รับวิตามินอีและ/หรือร่วมกับวิตามินซี (กลุ่มที่3-5) พบว่าปริมาณผลึกดังกล่าวในปัสสาวะมีจำนวนที่ลดลง การได้รับวิตามินอีและ/หรือวิตามินซีสามารถช่วยรักษาระดับการทำงานของเอนไซม์ซุปเปอร์ออกไซด์ ดิสมิวเทสในไต เมื่อเทียบกับกลุ่มที่ 2 นอกจากนี้พบว่าระดับของโปรตีนโคลโธในเลือด และไตของกลุ่มที่ 2 มีระดับลดลง แต่ในกลุ่มที่ได้รับวิตามินอีและ/หรือวิตามินซีสามารถเพิ่มระดับโปรตีนดังกล่าว นอกจากนั้นการศึกษาทางจุลพยาธิวิทยา พบว่าในกลุ่มที่ได้รับวิตามินอีและ/หรือวิตามินซี สามารถป้องกันความเสียหายที่เกิดขึ้นต่อเนื้อเยื่อไต ทั้งส่วนกลอเมอรูลัสและส่วนท่อไตได้ เมื่อเปรียบเทียบความเปลี่ยนแปลงที่เกิดขึ้นที่ไตในกลุ่มที่ 2 อย่างไรก็ดีเมื่อเปรียบเทียบพารามิเตอร์ส่วนใหญ่ที่ทำการศึกษาในครั้งนี้ พบว่าในกลุ่มที่ 5 มีความสามารถในการรักษาการทำหน้าที่ของไตได้มากที่สุด ดังจะเห็นได้จากความสามารถในการรักษาระดับของกลูตาไทโอนในไตได้

โดยสรุป หนูทดลองที่มีภาวะออกซาเลตในปัสสาวะสูง ทำให้การทำงานของไตลดลง เกิดการเปลี่ยนแปลงของความเครียดออกซิเดชัน และระดับของโปรตีนโคลโธ และเมื่อได้รับวิตามินอีและ/หรือวิตามินซี สามารถรักษาการทำหน้าที่ของไตรวมถึงป้องกันความเปลี่ยนแปลงทางจุลพยาธิวิทยาโดยอาศัยกลไกการเป็นสารต้านอนุมูลอิสระ โดยเฉพาะอย่างยิ่งเมื่อได้รับวิตามินสองชนิดร่วมกัน

ภาควิชา สรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สรีรวิทยาการสัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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ORAPUN JATURAKAN: Protective effects of combined vitamin E and vitamin C supplementation on renal functions and Klotho protein levels in hyperoxaluric rats. ADVISOR: PROF. CHOLLADA BURANAKARL, D.V.M., Ph.D., CO-ADVISOR: PROF. NARONGSAK CHAIYABUTR, D.V.M., Ph.D., ASST. PROF. THASINAS DISSAYABUTR, M.D., Ph.D., 172 pp.

Effects of combined vitamin E and vitamin C supplement on renal functions, oxidative stress status and Klotho protein levels in hydroxy-l-proline (HLP) induced hyperoxaluria in rats were investigated for 21 days' duration. The experimental study was divided into two parts; part I and part II. The seventy male Sprague Dawley rats were divided randomly into 5 groups: control (group 1), hyperoxaluric rats (group 2), hyperoxaluric rats with vitamin E supplement (group 3), hyperoxaluric rats with vitamin C supplement (group 4) and hyperoxaluric rats with vitamin E and C supplement (group 5). Hyperoxaluria was induced by feeding hydroxyl-L-proline (HLP) dissolved in drinking water. Intraperitoneal 200 mg/kg of vitamin E was given twice a week in group 3 and 5 while 500 mg of vitamin C was injected intravenously once a week in group 4 and 5. At the end of the study, clearance study was performed in the part I of the study while the vascular perfusion fixation was performed in the part II of the study for collection of the kidney tissues on day 21.

At the end of the study, the results showed that the urinary excretion of oxalate was significantly higher in HLP-treated group when compared with a control group ($p<0.05$) while it was maintained in group 3 and 5. The glomerular filtration rate (GFR), water and sodium reabsorption at the proximal tubule were drastically decreased in group 2 and improved considerably in group 3, 4 and 5. The total antioxidant status in urine (UTAS) decreased while the levels of malondialdehyde in plasma (PMDA) were significantly increased in group 2 as comparable with group 1 and they were reversed after receiving antioxidant alone or in combination (group 3, 4 and 5). The urinary calcium oxalate crystals increased in group 2 and reduced after receiving vitamin E and/or C (group 3-5). Additionally, vitamin E and/or C stabilized the activity of superoxide dismutase (SOD) in the kidney tissue, as comparable with group 2. Plasma and kidney Klotho protein were reduced significantly in group 2 while the vitamin E and/or C was improved the levels of Klotho protein in the plasma and kidney tissues. Furthermore, the histopathology demonstrated that vitamin E and/or C could preserved structural damage of glomerular and tubular part of kidney as compared to group 2. The most improvement of all parameters were seen in group 5 while receiving vitamin E and C which was supported by maintaining kidney GSH level when receiving HLP.

It is concluded that in rats with hyperoxaluria induced by HLP had diminished on renal functions, enhanced oxidative stress status and, lowered Klotho protein levels. Vitamin E and/or C can preserve the kidney functions and histopathology lesions with increased kidney Klotho protein expression especially when giving in combination which may be mediated by antioxidant effects.

Department: Veterinary Physiology
Field of Study: Animal Physiology
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
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# LISTS OF ABBREVIATIONS

| | |
|---|---|
| ADAM | A disintegrin and metalloproteinase domain-containing protein family |
| A-II | Angiotensin II |
| BP | Blood pressure |
| BUN | Blood urea nitrogen |
| Ca | Calcium |
| CaOx | Calcium oxalate |
| CAT | Catalase |
| CE | Capillary electrophoresis |
| $C_{H2O}$ | Free water clearance |
| $C_{In}$ | Clearance of inulin |
| Cit | Citrate |
| Cl | Chloride |
| $C_{Li}$ | Clearance of lithium |
| COD | Calcium oxalate dihydrate |
| COM | Clacium oxalate monohydrate |
| $C_{Osm}$ | Osmolar clearance |
| $C_{PAH}$ | Clearance of para-amino hippuric acid |
| Cr | Creatinine |

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| | |
|---|---|
| DHA | Dehydro-ascorbic acid |
| EG | Ethylene glycol |
| ER | Endoplasmic reticulum |
| ERBF | Effective renal blood flow |
| ERPF | Effective renal plasma flow |
| FE | Fractional excretion |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GFR | Glomerular filtration rate |
| GSH | Glutathione |
| Hct | Hematocrit |
| HLP | Hydroxy-L-proline |
| ICP-OES | Inductively coupled plasma atomic emission spectrometry |
| K | Potassium |
| LiCl | Lithium chloride |
| MAP | Mean arterial blood pressure |
| MDA | Malondialdehyde |
| Mg | Magnesium |
| mRNA | Messenger ribonucleic acid |
| Na | Sodium |
| Osm | Osmolarity |

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| | |
|---|---|
| Ox | Oxalate |
| PAH | Para-amino hippuric acid |
| PBS | Phosphate buffer solution |
| PCr | Plasma creatinine |
| PE | Polyethylene |
| PMDA | Plasma malondialdehyde |
| ROS | Reactive oxygen species |
| SBP | Systolic blood pressure |
| SOD | Superoxide dismutase |
| TAS | Total antioxidant status |
| UCr | Urine creatinine |
| UMDA | Urinary malondialdehyde |
| USG | Urine specific gravity |
| UTAS | Urinary total antioxidant status |
| $U_xV$ | Urinary excretion of substances x |
| V | Urine flow rate |
| Vit C | Vitamin C |
| Vit E | Vitamin E |
| Zn | Zinc |

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# CHAPTER I

# INTRODUCTION

Kidney stone disease and its complication have been discovered for long time ago. There are many biomolecules play a key role in the processes of the stones composites and its formation. Calcium oxalate (CaOx) stone is the most commonly type of the stone which found in both human and animals (Schubert, 2006; Tosukhowong et al., 2007; Sithanukul et al., 2010). Moreover, the recurrence rate of CaOx was high, approximately 50% within ten years (Uribarri et al., 1989). It is hypothesized that CaOx stone stimulated the formation of reactive oxygen species (ROS) (Khan, 2013) and the deterioration of kidney functions in both animal and human (Huang et al., 2003; Huang et al., 2006; Hirose et al., 2010; Ma et al., 2013). Therefore, the mechanisms of renal impairment may involve the overwhelming of ROS products leading to imbalance of redox homeostasis.

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The anti-oxidant therapy has been reported for reduction of kidney tissue injury from ROS in CaOx stone and hyperoxaluria state. The supporting therapy with vitamin E in hyperoxaluric patients demonstrated the reduction of urinary risk factors (Anbazhagan et al., 1999). Vitamin E also prevented the new formation of stone by promoting the expression of stone inhibitor molecules (Sumitra et al., 2005). In hyperoxaluric rats induced by ethylene glycol, receiving vitamin E daily decreased ROS and oxalate excretion (Thamilselvan and Menon, 2005; Huang et al., 2006; Huang et

al., 2009). Therefore, the reduction of ROS especially from lipid peroxidation may be a crucial role in the prevention of renal impairment and CaOx stone formation.

Ascorbic acid or vitamin C is another potent water-soluble ROS scavenger. Vitamin C can be recycling oxidized form of glutathione to reduced form of glutathione, which is one of the major antioxidant biomolecule in cells (Braun et al., 1996). Previous report revealed that vitamin C improved oxidative status in kidney injury in animal model (Mustacich et al., 2007). However, the usefulness of vitamin C in CaOx kidney stone disease is controversy due to its metabolite as a source of endogenous oxalate production (Yaich et al., 2014).

Interestingly, evidences exist of the vitamin E and vitamin C had a synergistic and cooperative effects (Niki et al., 1985; Niki et al., 1995). For that reason, the combination of vitamin E and C could provide more potent antioxidant effects than giving alone. The study in gentamicin induced nephropathic rats revealed that the combination of vitamin E and C could protect kidney injury and ameliorated the reduction in glomerular filtration rate (Kadkhodaee et al., 2005). Moreover, the study in LLC-PK1 proximal tubular cells exposed to urinary oxalate showed reduction in oxidative injury after combination treatment (Thamilselvan et al., 2014). For this reason, the combination of vitamin E and vitamin C might improve anti-oxidant status in patients with hyperoxaluric state and CaOx stone.

The klotho gene was first discovered for an anti-aging functions while the location of this protein was found more than 90% in the kidney (Kuro-o et al., 1997).

Klotho protein could be found in the extra-renal tissue such as parathyroid gland (Kuro-o et al., 1997) and vascular epithelium in human (Markiewicz et al., 2016). There are three types of Klotho protein; full length transmembrane, soluble and secreted forms. An important function of transmembrane form of Klotho protein involved the regulation of Ca and inorganic phosphate metabolism in the kidney (Razzaque, 2009; Farrow and White, 2010; Kim et al., 2015). In addition, the soluble form is detected in CSF, plasma and urine (Yamamoto et al., 2005) which plays a significant role in regulation of many transporter proteins including calcium channel, potassium channel and Na/K ATPase in the kidney (Huang, 2010; Sopjani et al., 2011). Moreover, soluble Klotho function can act as a hormone and have endocrine function by discovered its receptor (Dalton et al., 2017). Because of these important roles of Klotho protein, it is not surprising that Klotho protein controlled many fundamental mechanisms in the whole body.

Recently, a single nucleotide polymorphisms (SNPs) of G395A in *klotho* gene was found in CaOx stone patients (Telci et al., 2011). Therefore, Klotho protein may be altered in kidney stone diseases and could be used as a biomarker in animals and human who are suffering from CaOX urolithiasis. However, there was no data demonstrated the level and the role of Klotho protein in calcium oxalate stone or hyperoxaluric condition. Furthermore, the preventive effects of antioxidants in hyperoxaluria in relation to Klotho protein has not yet been investigated.

Therefore, the objectives of the present study were

1. To investigate the changes in renal hemodynamic, renal handling of electrolytes and urinary supersaturation in relation to hyperoxaluria induced by hydroxy-L-proline (HLP).

2. To investigate the changes in Klotho protein during hyperoxaluric condition.

3. To investigate the protective effects of combination of vitamin E and vitamin C supplement on renal hemodynamics, renal handling of electrolytes, renal oxidative stress, urinary supersaturation.

4. To investigate the combination of vitamin E and vitamin C on Klotho protein levels in HLP induced hyperoxaluric rat.

5. To study the correlations between Klotho protein levels, oxidative stress and renal function in HLP-induced hyperoxaluric rats after supplement with combination of vitamin E and vitamin C.

The hypotheses of this study were

1. Hyperoxaluria induced by HLP impairs renal hemodynamic, renal handling of electrolytes and urinary supersaturation.

2. The Klotho protein level is lower in hyperoxaluric rat than control group.

3. The combination of vitamin E and vitamin C supplement alleviates renal hemodynamics, renal handling of electrolytes, renal oxidative stress, urinary supersaturation in hyperoxaluric rat.

4. The combination of vitamin E and vitamin C supplementation increases Klotho protein level in hyperoxaluric rats.

5. Klotho protein levels correlate with oxidative stress and renal function in HLP induced hyperoxaluric rat after supplement with combination of vitamin E and vitamin C.

Therefore, this study would provide more useful information for pathophysiology and mechanism of hyperoxaluria and CaOx stone. Moreover, the data from the present study could be applied using antioxidant, vitamin E and/or vitamin C to protected the damage of kidney tissue from ROS and to reduce the recurrence rate of CaOx stone. Importantly, Klotho protein could be an early screening biomarker for stone formation and predictive biomarker for kidney injury beyond hyperoxaluria and CaOx stone condition.

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# CHAPTER II

# REVIEWS OF LITERATURE

## A. Calcium oxalate nephrolithiasis

Nephrolithiasis or a kidney stone disease has become problem in medicine in both human and animals for long time ago. There have been widely studies in the pathogenesis and treatment protocol for the kidney stone. However, several new theories of mechanisms and molecules that involve in the urolithiasis were recently reported. The type of kidney stone can be clarified by its composition, and the most prevalent type in human is calcium oxalate (CaOx) kidney stone (Schubert, 2006). Importantly, CaOx stones have high recurrence rate, which was recurred up to 35-50% within ten years (Uribarri et al., 1989). It also deteriorates renal functions (Worcester et al., 2003; El-Zoghby et al., 2012). The complication of CaOx stone such are severe pain at the flank area, acute kidney injury and urinary tract obstruction, hydronephrosis and chronic kidney disease (CKD) were also developed even though the stones were removed. Several factors are associated with pathogenesis of CaOx stone formation such as genetics, food, promoters, inhibitors, urine pH and urinary supersaturation (Thongboonkerd et al., 2006). The successfulness of treatment after the removal of the stone depends on the correction of all known causes and risk factors of CaOx stone formation in each patient and maintenance of the low-risk diet.

## B. The physicochemical role of kidney stone formation

The mechanisms of stone formation were associated with urinary supersaturation, stone promoters, stone inhibitors and crystal-cell interaction (Khan, 2004). Firstly, the supersaturation of urine caused crystallization of crystalline particles in the urine. The mechanisms of classical crystallization processes are composed of nucleation, crystal growth and aggregation (Saw et al., 2008). Secondly, the forming crystal may pass through the urine as crystalluria or is endocytosed by the renal epithelial cell and then transported to renal interstitium as called " crystal-cell interaction" (Khan, 2004). Lastly, the crystal growth, aggregation and retention in the upper or lower urinary tract are contributed for stone formation. The supersaturated urine occurred when the chemical driving force of dissolve solutes in the urine above the equilibrium point. The major solutes in the urine are ions and salts such as calcium, phosphate, magnesium, ammonium, urate and oxalate (Rodgers et al., 2011). The difference between the concentration of solution (Cs) and equilibrium (Cq) is concentration driving force ($\Delta C$) as shown in the equation below:

$$\Delta C = Cs - Cq$$

The supersaturation ratio (S) of the urine is

$$S = Cs/Cq$$

To further investigate into the prediction of urine crystallization, computerized ion-activity product was studied by Tiselius et al. (2003) and named Tiselius index. In rat urine, the ion-activity product of calcium oxalate ($AP_{CaOx}$) was defined as:

$$\frac{4067 * \text{Calcium}^{0.93} * \text{Oxalate}^{0.96}}{(\text{Citrate} + 0.015) * \text{Magnesium}^{0.55} * V^{0.99}}$$

The factor 4067 and 0.015 were derived from computerized program and the factor V is urine volume in 24 hours. However, stone formation, is not only depended on the supersaturation of urine but also accompanying with the presence of stone promoters and inhibitors. The stone promoters and inhibitors could be any ions or molecules that promote or disturb the formation of stone nucleation, crystallization and cell-adhesion. Thus, the mechanisms of stone formation are very complicated and need to be further investigated.

## C. Oxidative stress and redox cycle relating to CaOx

Oxidative stress is an imbalance and/or the loss of homeostasis between pro-oxidants and anti-oxidants in the body due to enhanced free radical production or diminished of the free radical scavenging system (Mandelker, 2008). In homeostasis of redox cycle, the living organisms can generate the numerous superoxide anion ($O_2^{\bullet}$) from enzymatic (xanthine oxidase and NADPH oxidase) and non-enzymatic pathways (mitochondria and endoplasmic reticulum) (Mandelker, 2008) as shown in Figure 2-1. The radical molecules are the molecules that have un-paired valence electron on their last electron shell and are unstable molecules which can easily form dimerization or polymerization with each other. The free radical molecules attack the others resulting

in conformational changes and may loss of their function. These oxidized molecules can damage cells and interfere to the normal function of the tissues and organs.

CaOx damaged renal epithelial and tubular cells and increased the lipid oxidation in both in vitro and in vivo studies (Huang et al., 2009; Hirose et al., 2010). Malondialdehyde (MDA) ($C_3H_4O_2$), volatile and short-chain dicarbonyl compound, is an end product of lipid oxidation (LPO) (Girotti, 1985). In hyperoxaluria, over production of ROS induced renal epithelial cells injury leading to LPO reaction, MDA production resulting in conformational change of cell membrane (Selvam, 2002). Damaged lipid bilayer promoted crystal attachment, retention and/or tubular stone formation (Selvam, 2002). The in vitro study showed that incubation of proximal tubular cell line with oxalate increased MDA production while giving the free radical scavengers reduced MDA levels (Rashed et al., 2004). In ethylene glycol (EG)-induced CaOx nephrolithiasis rats, the kidney MDA levels was associated with CaOx crystal deposition in the kidney (Thamilselvan and Menon, 2005). Moreover, patients with CaOx stones had urine and red blood cell MDA concentrations higher than normal volunteers (Ma et al., 2013). Regarding this, the levels of MDA could be used as a biomarkers and therapeutic index of renal tubular injury from CaOx crystal formation and retention.

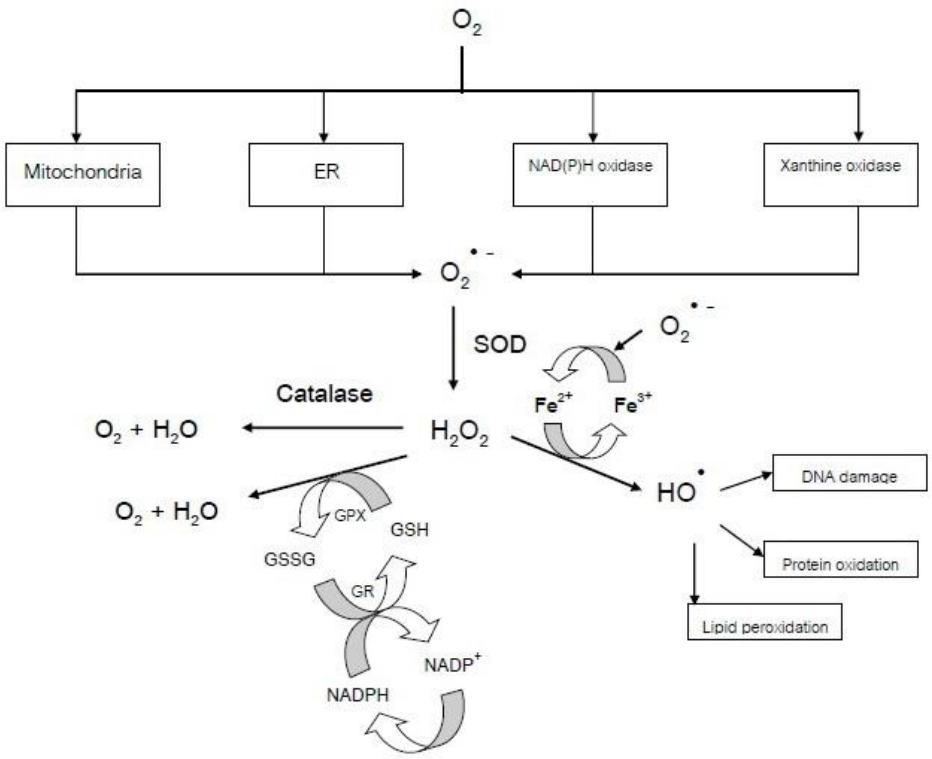


**Figure 2-1** The redox cycle. The equilibrium between the production of free radical ($O_2^{\bullet}$, $H_2O_2$ and $OH^{\bullet}$) and free radical scavenging system (SOD; superoxide dismutase, catalase, GPX; glutathione peroxidase, GR; glutathione reductase, GSH; reduced glutathione and GSSG; oxidized glutathione).

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## D. CaOx therapy and recurrence rate

Potassium citrate or thiazide diuretics supplementation after stone removal can reduce CaOx recurrence and renal injury from oxidative stress (Thamilselvan et al., 2003). Hence, the combination therapy between lime powder, potassium citrate, pH modification substances or prescription diet and antioxidants should be used to reduce recurrence rate and renal injury from urolithiasis and preserved the normal nephron.

## E. Hydroxy-L-proline, ethylene glycol and animals model for the study of CaOx stone

The trans-4-hydroxy-L-proline (HLP) (2S, 4R)-4-Hydroxypyrrolidine-2-carboxylic acid ($C_5H_9NO_3$) (Figure 2-2) has molecular weight of 131.13 g/mol. The HLP is a precursor of oxalate. It is degraded and excreted from the body without reutilized for protein synthesis. It also causes a toxic by-product accumulating in many organs (Jiang et al., 2012).

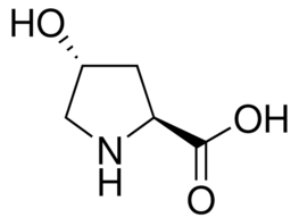


**Figure 2-2** The chemical structure of trans-4-hydroxy-l-proline (HLP)

The breakdown of HLP generates pyruvate ($C_3H_4O_3$) and glyoxylate ($C_2H_2O_3$) (Knight et al., 2006). The glyoxylate can be metabolized to glycine, glycolate and oxalate by enzyme alanine: glyoxylate aminotransferase (AGT), glyoxylate reductase (GR) and lactate dehydrogenase (LDH), respectively (Knight et al., 2006). The LDH is the rate limiting step enzyme for oxalate generation (Holmes and Assimos, 1998). Plasma oxalate could be derived from the others source such as diet and vitamin C (Holmes and Assimos, 1998). Oxalate, the final metabolic waste, must be eliminated in the urine and feces (Marengo and Romani, 2008). Oxalate does not bind with any plasma protein and freely filtrated through the glomeruli (Marengo and Romani, 2008). In the proximal

tubule, the oxalate is excreted using sulfate anion transporter 1 family including SLC26A6 and SLC26A1 which are located in apical and basolateral membrane, respectively (Marengo and Romani, 2008).

## F. HLP and hyperoxaluria

Hyperoxaluria is defined as the amount of oxalate in the urine that exceeds 40-45 mg/day in healthy adults' human (Borghi et al., 1999). It has been shown that giving high dose of HLP (5% w/w) into regular chow could induce hyperoxaluria and CaOx urolithiasis in rats (Khan et al., 2006). Tawashi et al. (1980) found that the intraperitoneal injection of HLP at dose 2.5 mg/kg BW in rats caused CaOx crystal formation in renal tissue and kidney swelling within 24 hours after receiving HLP. Moreover, giving 2 % w/v of HLP in drinking water caused CaOx crystal retention in renal tubule and hyperoxaluria in Dalh salt-sensitive rats (Wiessner et al., 2009). In the past, EG was widely used to induce hyperoxaluria and CaOx nephrolithiasis in rat model (Yamaguchi et al., 2005; Li and McMartin, 2009). However, the use of EG-induced hyperoxaluria caused kidney tissue injury and renal function impairments from toxic metabolites of EG besides CaOx crystal (Bacchetta et al., 2009). For this reason, the HLP-induced hyperoxaluria and CaOx crystal might be superior to use as a model for study the effects of hyperoxaluria and CaOx crystal on renal injury.

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## G. The concept of lithium clearance study

Lithium is an alkali metal element in group IA of periodic table which has atomic number and atomic mass smaller than sodium and potassium (atomic number and mass of Li, Na and K are 3, 11, 19 and 6.941, 22.989 and 19, 39.098, respectively). In Nephrology study, lithium has been used for treatment of bipolar disorders by Food and Drug Administration of USA (FDA) (Morimoto et al., 2005). Moreover, the clearance study of lithium has been reported for assessment of water and sodium transportation from proximal tubule of the kidney (Thomsen and Shirley, 1990; Thomsen and Shirley, 1997).

Lithium has no specific receptor and used the same transporter protein similar to sodium which is amiloride-sensitive Na channel (ENaC) (Thomsen and Shirley, 2006). From the study of lithium transport, it was found that lithium is freely filter through the glomerulus in the same manner of sodium. The micro-puncture study of ultrafiltrate from glomeruli found that lithium was reabsorbed in the same manner of the water and sodium in proximal tubule (Hayslett and Kashgarian, 1979). Importantly, there are no lithium reabsorbed in distal tubular compartment in normal physiological condition of nephron. So, the clearance of lithium could be demonstrated correctly of water and sodium transport in proximal tubule (Thomsen and Shirley, 2006).

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## H. Oxalate and renal handling of oxalate

Oxalate, metabolic waste of the cell, generated in the body by two major sources, endogenous oxalate and exogenous oxalate. The critical role of oxalate metabolism is formation of CaOx kidney stone that was revealed by deletion of oxalate transporter in mice could cause hyperoxalemia and hyperoxaluria leading to CaOx kidney stone (Jiang et al., 2006). The food enriches of oxalate is an important exogenous source of oxalate (20-40%) which reabsorbed via gastrointestinal tract and excreted mainly through renal excretion (Hatch and Freel, 2003). On the other hand, liver metabolism is endogenous source of oxalate via oxidation of (Coulter-Mackie and Lian, 2006). After oxalate ingestion, oxalate was absorbed from intestinal tract via solute carrier family 26A (SLC26A) transporter protein (Hatch and Freel, 2003; Jaeger and Robertson, 2004). The study using $14^{C}$-oxalate found that intestinal epithelium reabsorbed only free oxalate ion (Hatch and Freel, 2008). Calcium and magnesium in intestinal tract attached oxalate ion directly while binding oxalate cannot reabsorb from gut to plasma (Marengo and Romani, 2008). However, elevated levels of lipid in intestinal attached calcium leading to low levels of calcium and subsequently increased oxalate reabsorption (Emmett et al., 2003).

## I. Renal handling of oxalate

In glomerulus, oxalate was freely filter. In rat, similar creatinine clearance and oxalate distribution was formed compared with human (Osswald and Hautmann, 1979;

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Boer et al., 1984; Sugimoto et al., 1993). Study used clearance of inulin together with $14^{C}$-oxalate in human found that the half-life of oxalate was 97 minutes while the excretion into urine was higher than inulin (2.31±0.05 fold) (Osswald and Hautmann, 1979). The same research group conclude that oxalate enters the tubular fluid and excreted into the urine by glomerular filtration and extra tubular transport. The transporter protein in each parts of nephron was proposed (Figure 2-3 and 2-4).

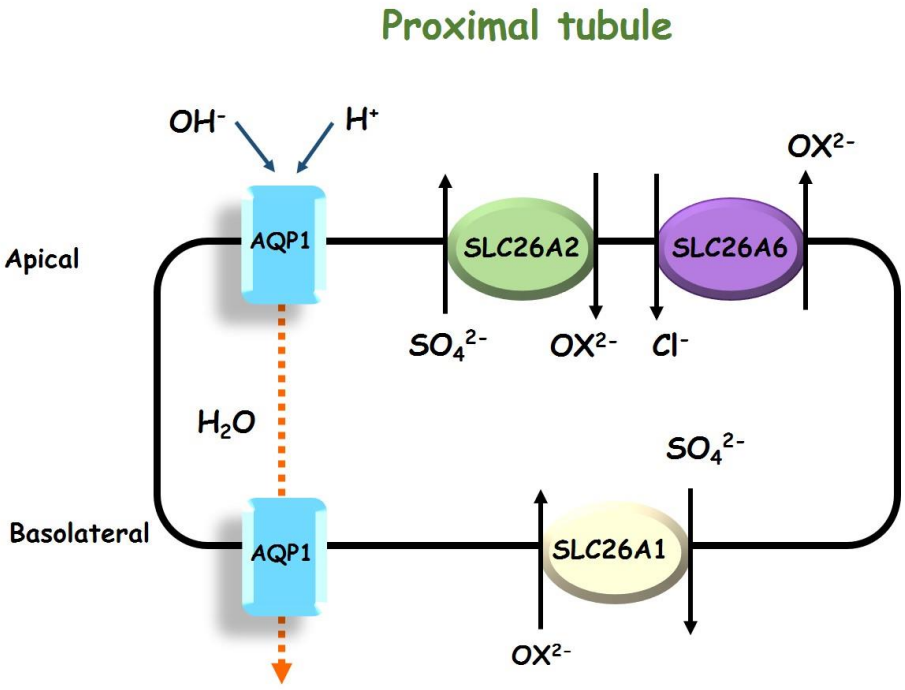


**Figure 2-3** Oxalate in ultrafiltrate is mediated through several types of SLC26 transporter. Apical side, oxalate was transported into tubular cell by SLC26A2 and exchanged with intracellular $SO_4^{2-}$ while the SLC26A6 transported $Cl^-$ into the cell and exchanged with intracellular oxalate. Basolateral side, oxalate from plasma was transported into tubular cell by SLC26A1 and exchanged with intracellular $SO_4^{2-}$.

In tubular compartment of nephron, oxalate was mediated through many types of SLC26 transporter protein. Proximal tubule, both convoluted and strength parts

expressed SLC26A2 and SLC26A6 at the apical membrane while the basolateral membrane expressed SLC26A1 (Robijn et al., 2011). However, oxalate could be exported from tubular cells into tubular lumen throughout the distal part of nephron including distal tubule, thick ascending limb and outer medullary collecting duct by SLC26A6 (Figure 2-4). Importantly, it is postulated that the tubular reabsorption of oxalate does not occur, practically. It is hypothesized that the high levels of plasma oxalate caused the higher oxalate levels in ultrafiltrate throughout the tubular fluid and then enhanced supersaturation of urine and CaOx crystal formation.

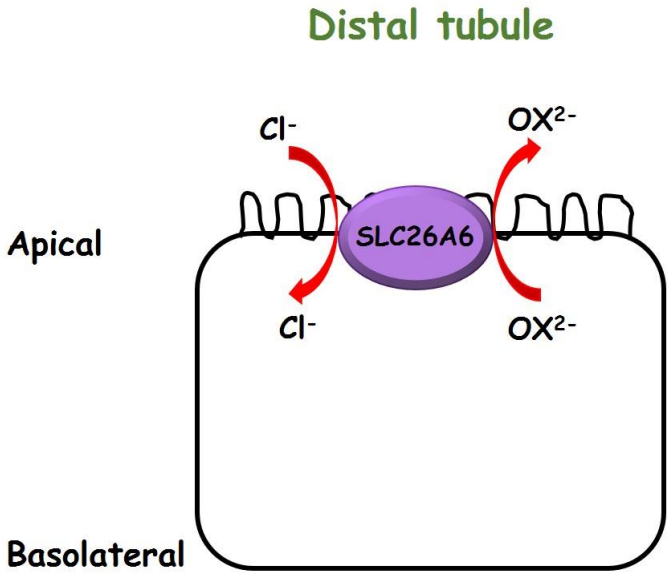


**Figure 2-4** Oxalate in distal tubule is mediated via SLC26A6 transporter. Apical side, oxalate was only exported by SLC26A6. $OX^{2-}$ ; oxalate, $Cl^-$; Chloride

## J. Citrate and renal handling of citrate

Citrate is an important biomolecule for renal metabolism in controlling acid-base balance. Moreover, citrate is a central molecule in tricarboxylic acid cycle which

is an important energy source of renal cell especially for proximal tubular cell (Hamm, 1990). Additionally, citrate in urine chelated the ionized-calcium and reduced binding of oxalate and free ionized calcium (Ohana et al., 2013). Citrate has three forms of tricarboxylic acid including $Cit^{-1}$, $Cit^{-2}$, and $Cit^{-3}$ which depend on pH and pKa. It was reported that a trivalent citrate ($Cit^{-3}$) is found mainly in plasma (Minisola et al., 1989). Kidney is the major organ for controlling the levels of citrate (Simpson, 1967). In plasma, the concentration of citrate is normally low and it is formed the complex with another ion rapidly such as calcium, magnesium and sodium (Walser, 1961). Citrate is freely filter through the glomerulus and then reabsorbed mainly in proximal tubule (Grollman et al., 1963) (Figure 2-5). It was reported that systemic pH and luminal pH in tubular lumen affected transportation of citrate into renal tubular cell. The pH elevation of luminal fluid decreased tubular reabsorption of citrate due to its increased the levels of cellular citrate (Hamm, 1990).

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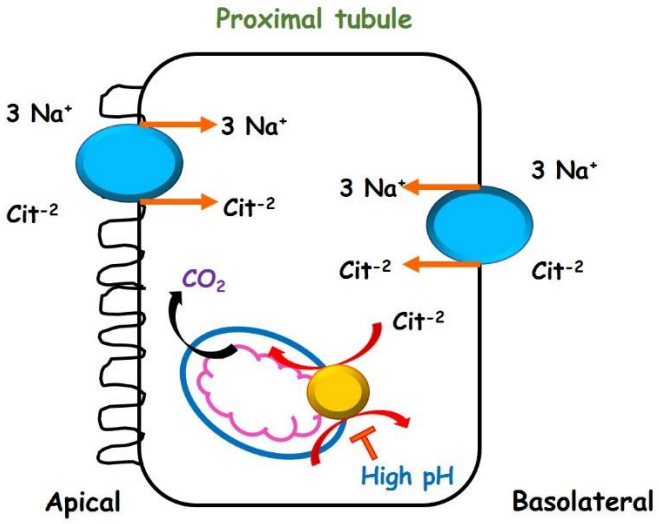


**Figure 2-5** Proximal tubular cell transported citrate ($Cit^{-2}$) into the cell via sodium citrate cotransporter or $Na^{+}$-dependent dicarboxylate co-transporter (NaDC-1).

## K. Interaction between citrate and oxalate

Citrate could play a crucial role in inhibition of oxalate anion binding with ionized calcium in luminal tubular fluid which proposed by Ohana and co-workers (2013). There interaction between NaDC-1 (citrate transporter) and SLC26A6 (oxalate transporter) was studied (Ohana et al., 2013). The SLC26A6 inhibits NaDC-1 while NaDC-1 activates SLC26A6 leading to retention of citrate in luminal tubular fluid. After that, oxalate had higher affinity to bind with citrate than ionized calcium. The complex of oxalate and citrate had high water solubility while calcium-oxalate cannot dissolve easily in water.

## L. Vitamin E (alpha-tocopherol): nomenclature, chemical structure and functions

Vitamin E or alpha-tocopherol was discovered in 1922 by Evans and Bishop (Evans and Bishop, 1922; Evans, 1962). Vitamin E ($C_{29}H_{50}O_2$, molecular weight 430.7 g/mol, IUPAC Name = (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol) composed of major two groups in molecule. The first one is aliphatic side chain which is located next to the hydrophilic layer of cellular membrane (Figure 2-6) while the chromanol ring is in the inner of lipid bilayer and reacted as free radical scavenger in hydrophobic condition (Niki et al., 1985). Tocopherol is types and names according to the difference of methyl group at carbon atom, C5; C7; C8 on chromanol ring which described in Table 2.1. The alpha (α) type of tocopherol which containing three methyl groups is the most biological active form.

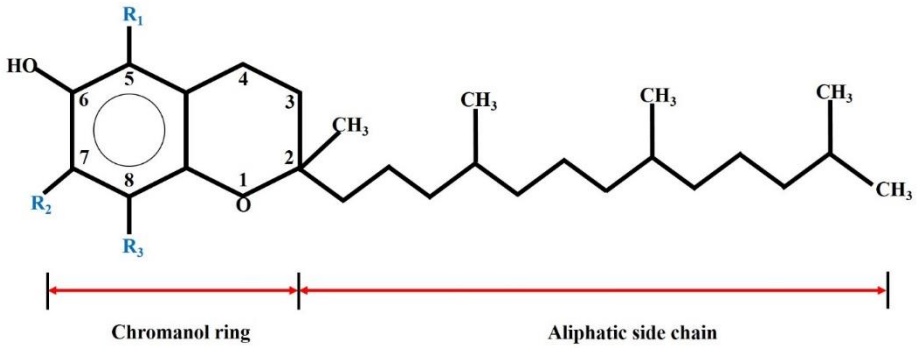


**Figure 2-6** The chemical structure of alpha- tocopherol which compose of hydrophobic chromanol ring and hydrophilic aliphatic side chain.

**Table 2.1** The different type of tocopherol categorized by using the difference type of methyl group on carbon atom in chromanol ring

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| α | $CH_3$ | $CH_3$ | $CH_3$ |
| β | $CH_3$ | H | $CH_3$ |
| γ | H | $CH_3$ | $CH_3$ |
| δ | H | H | $CH_3$ |

Vitamin E is the highly potent capacity for antioxidant by scavenged a peroxyl radical. Moreover, vitamin E maintained the integrity of lipid bilayer of all cells in the body and the integrity of lipid raft molecule. It is reported that the hydroxyl group at the 6th carbon atom of chromanol ring is the reduction site when vitamin E scavenged the free radical in lipid peroxidation and transformed to tocopheroxyl radical (Vit E-O$^{\bullet}$) (Figure 2-7) (Serbinova et al., 1991; Suzuki et al., 1993). ROS and reactive nitrogen species (RNS) attacked the polyunsaturated fatty acids (PUFA) which is the major component of cell membrane resulting in PUFA$^{\bullet}$ radical. After that, PUFA$^{\bullet}$ radical was rearranged and then attacked by oxygen yielding a peroxyl radical (PUFAOO$^{\bullet}$). Importantly, a peroxyl radical is very sensible for propagation of chain reaction resulting in auto-oxidation of the other PUFA molecules. Hence, vitamin E, a fat-soluble antioxidant, is major molecules which plays a crucial role for termination of PUFA auto-oxidation in cell membrane (Figure 2-7). Vitamin E donated a H atom to PUFA$^{\bullet}$ radical

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yielding unpaired electron and delocalized in chromanol ring of tocopheroxyl radical (Vit E-O•). Additionally, the proportion between vitamin E in cell membrane and phospholipids is only 1 molecule per 2000-3000 molecules of phospholipids (Zingg, 2007). In the in vivo environment, a radical vitamin E, is rapidly regenerated to active vitamin E and recycling by using both water soluble antioxidants and intracellular antioxidant enzymes.

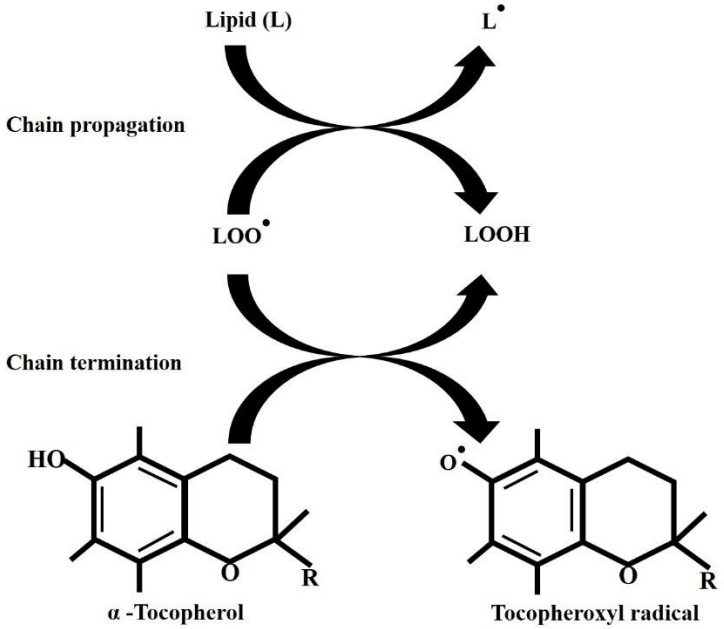


**Figure 2-7** Vitamin E scavenges the free radical (O•) from lipid peroxidation and then transforms to tocopheroxyl radical.

## M. Transport, absorption and cellular uptake of alpha-tocopherol

Since vitamin E is fat soluble, the mechanism of transport, cellular uptake and excretion are close to lipophilic molecules with lipid-rich nutrients. After consumption of fat-containing food, it is digested by lipoprotein lipase (LPL) to micelles which contained vitamin E and then transports into the intestinal cells and subsequently

lymphatic system (Borel et al., 2013). Vitamin E is transferred with chylomicron, high density lipoproteins (HDL) and phospholipid transfer protein (PLTP) (Reboul and Borel, 2011). The HDL-containing vitamin E is taken up by the hepatocytes and is rearranged and transported to plasma with very low-density lipoproteins (VLDL) for distribution to peripheral tissue (Reboul and Borel, 2011). Tissue accumulations of vitamin E are depending on α-tocopherol transfer protein (α-TTP) and the regulation of vitamin E metabolism and excretion by the liver (Herrera and Barbas, 2001).

## N. Distribution of intracellular vitamin E

After vitamin E is transported from lymph to blood stream by chylomicrons and chylomicron remnant. Peripheral tissues receive vitamin E from chylomicrons by using lipoprotein lipase. Afterward, vitamin E which couple with chylomicrons, LDL or HLDL are uptake to the hepatocytes by using several proteins such as scavenger receptor class B type I (SR-BI) and low-density-lipoprotein receptor (LDLR). In cellular compartment, α-tocopherol transfer protein (α-TTP), tocopherol binding protein (TBP) and tocopherol-associated protein (TAP) transport alpha-tocopherol while other vitamin E isoforms including β-, γ-, and δ-tocopherol and tocotrienols are metabolized by cytochrome P450 and then excreted through urine (Zingg, 2007).

## O. Metabolism of vitamin E

Until now, there are not clearly understood about the best final markers for true metabolites form of vitamin E (Schmölz et al., 2016). Many reported demonstrated

that Simon metabolites which compose of alpha-tocopheronic acid and alpha-tocopheronelactone (α-TL) are the side chain degradation of vitamin E and then secreted in the urine of human and mice (Pope et al., 2001). On the contrary, conjugate of α-TL could be a good biomarker for metabolite form of vitamin E and was elevated significantly in children who suffered from type 1 diabetes (Sharma et al., 2013). The initiation of tocopherol metabolism in liver is originated by using cytochrome P4F2/P3A4 with dependent of ω-hydroxylation followed by β-oxidation. Finally, the end products from vitamin E metabolism are carboxyethylhydroxychroman (CEHC), 12'-hydroxy-chromanol and 11'-hydroxy-chromanol (Schultz et al., 1995) which excreted through urine and feces of human and mice (Bardowell et al., 2012). It is postulated that the control of vitamin E metabolism is a major role for the regulation of bioavailability of active vitamin E.

## P. Bioavailability and plasma kinetics of vitamin E

Bioavailability of vitamin E was influenced by many factors including vitamin E intake from food, vitamin E absorption via involving protein and molecules and metabolism of vitamin E (Schmölz et al., 2016). The half-life of alpha tocopherol is 48 hours (Traber, 2007).

## Q. Cellular signaling modulations activity and gene expression control by vitamin E

Controlling of the cellular signaling pathway by vitamin E using MetaCore analyzing for transcription regulatory networks in Madin-Darby bovine kidney epithelial cell line (MDBK)-treated with vitamin E was demonstrated (Li et al., 2010). The same research group found that vitamin E modulated both up-regulation and down regulation of functional networks. Vitamin E can down-regulate of cytoskeleton organization, RNA processing, cellular response to stress and development of immune system while vitamin E can up-regulated cellular component movement, organ development and increased regulation of biological process. Moreover, vitamin E could modulate specific enzymes which involved in cellular signal transduction such as activation of protein phosphatase 2A, diacylglycerol (DAG) kinase, protein tyrosine phosphatase, or inhibition of protein kinase C (PKC), PKB, phospholipase A2, cyclooxygenase, lipoxygenases and mitogen activated protein kinase (MAPK) signaling pathway (Zingg, 2007). It is noteworthy that vitamin E is a key biomolecule which plays many crucial roles for controlling the homeostasis.

## R. Vitamin E and renal injury

Renal injury can be presented from many mechanisms including ischemic reperfusion injury leading to over production of free radical. The in vivo study in rats with experimentally induced renal injury revealed that intraperitoneal injection of

vitamin E could reduce lipid peroxidation (Rhoden et al., 2001) and improve renal blood flow (RBF) (Thongchai et al., 2008) . The recovery of renal injury induced by **ω**-nitro-L-arginine was enhanced after vitamin E treatment with reduction of proteinuria. In addition, vitamin E alleviated glomerulosclerosis and reduced oxidative stress in nephrectomized rat (Tain et al., 2007). Using vitamin E-coated dialyser could reduce the levels of lipid peroxidation in patients with hemodialysis (Morimoto et al., 2005). However, Tasanarong and co-workers (2013) reported that oral supplementation of **α**-tocopherol at the dose of 350 mg / kg bodyweight in patients with contrast-induced acute kidney injury could not prevent kidney injury.

## S. Vitamin E, hyperoxaluria and CaOx nephrolithiasis

The anti-urolithic effects of **α**-tocopherol have been revealed in many studies. In vivo study showed that diet with low vitamin E levels can enhance the crystal formation via oxidative damage in ethylene glycol-induced hyperoxaluric rat model (Thamilselvan and Menon, 2005; Huang et al., 2009). The intraperitoneal injections of vitamin E at the dose of 8 mg/rat/week for 6 weeks improved antioxidant enzyme and could prevent the CaOx crystal deposition in the nephron (Huang et al., 2009). The effect was similar when given diet supplementation with vitamin E which could reduce the CaOx crystals retention in EG induced CaOx nephrolithiasis rats (Naghii et al., 2014).

## T. Vitamin C (ascorbic acid): nomenclature, chemical structure and functions

Vitamin C or ascorbic acid ($C_6H_8O_6$, IUPAC name; (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one, Figure 2-8) is a potent water-soluble hydrogen donor which plays an important role in regulation of many cellular metabolisms such as collagen synthesis, wound healing and antioxidants as previously reviewed by Jacob and Sotoudeh (2002). Its pivotal role of ascorbic acid is due to the properties of chemical structure known as a lactone ring (Figure 2-8 and 2-9). Most of mammals can synthesize vitamin C in the liver except human, non-human primates and guinea pigs (Du et al., 2012). Vitamin C is a highly effective intracellular scavenging agent since it could be oxidized rapidly one or two electrons (Mandl et al., 2009) can be reduced back to ascorbic acid using NADPH-dependent recycling (Banhegyi et al., 1997). In human, vitamin C has limited intestinal absorption from oral ingestion at 3 g per day (Rivers, 1989). Ingestion of 30 mg / day of vitamin C can exert the plasma levels up to 7 μM in male and 12 μM in female (Levine et al., 2011). The normal plasma vitamin C concentrations should not be lower than 4 μM to prevent scurvy (Delanghe et al., 2011). The intravenous administration of vitamin C caused more rapidly increase in the plasma level of vitamin C than oral ingestion (Padayatty et al., 2010). Furthermore, giving intravenous vitamin C caused lower urinary oxalate excretion than

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oral ingestion due to the lower oxalate absorption by the intestine (Linster and Van Schaftingen, 2007).

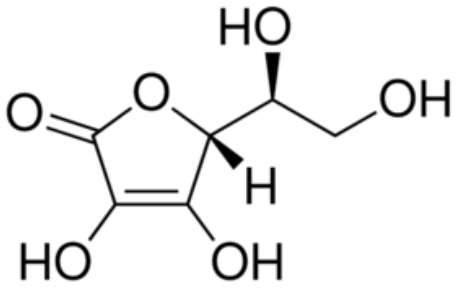


**Figure 2-8** The chemical structure of ascorbic acid.

## U. Antioxidant activity of vitamin C

The essential function of vitamin C is maintained redox homeostasis. The major organelles that generated the most oxidative respiration leading to ROS are mitochondria and endoplasmic reticulum. Consequently, vitamin C is a major potent water soluble free radical scavenger, it is plausible that vitamin C plays a key role in cytosol compartment of both organelles (Mandl et al., 2009). The reducing property of vitamin C has been revealed in Figure 2-9. In general, vitamin C could donate their electron to another enzyme, oxidant agents and other electron acceptors. Moreover, the superior property of vitamin C is its intermediate form, semidehydroascorbic acid, cannot be attacked by superoxide anion and DHA can be rapidly recycling in cellular compartment and turn back to ascorbic acid again.

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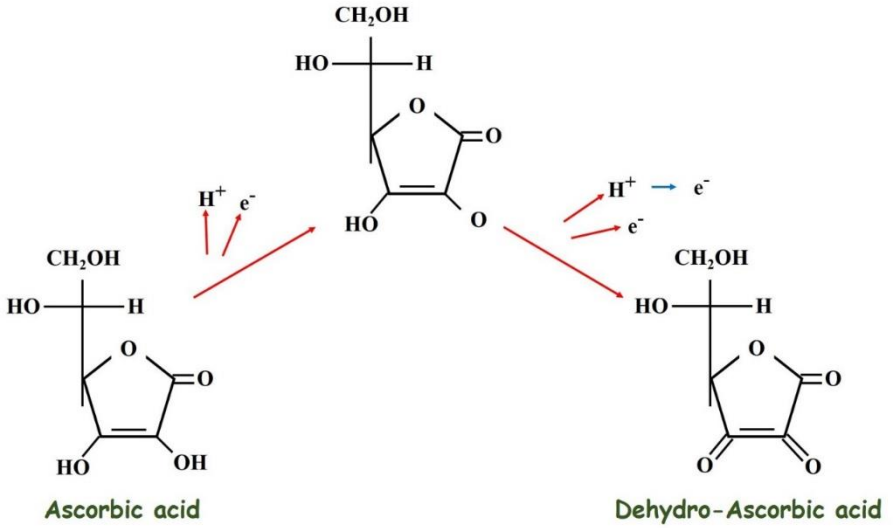


**Figure 2-9** The oxidation of vitamin C started with L-ascorbic acid donation one electron (semidehydroascorbic acid) and then rearrangement and follow by donation of two electrons and become oxidized form or dehydroascorbate

## V. Transport, absorption and cellular uptake of vitamin C

After receiving vitamin C, it was absorbed from the intestinal lumen and released into the bloodstream. The dose-to-plasma concentration relationship is reflected by saturation curve, attaining an initial steep and non-linear course, until steady-state is reached, defining plasma saturation at around 70 μM in humans (Levine et al., 1996). At doses over plasma saturation, urinary excretion is increased and oral bioavailability decreased, thereby sustaining steady-state equilibrium (Levine et al., 1996). Plasma vitamin C was uptake and reabsorbed into different tissues using different transporter systems which was saturated by the Michaelis-Menten absorption rate (Malo and Wilson, 2000; Wilson, 2002; Wilson, 2005).

Vitamin C was tightly regulated and controlled by bioavailability, accumulation and distribution in tissues, rate of utilization and recycling processes and renal reabsorption and excretion (Levine et al., 2011). The process involved cellular uptake and utilization of vitamin C including passive diffusion, facilitated diffusion, active transport and recycling (Wilson, 2005).

## W. Vitamin C recycling

After vitamin C was uptake by intestinal epithelium via SVCT1, vitamin C is released into the bloodstream and easily oxidized subsequently produced DHA rapidly then taken up to erythrocytes and neutrophils via GLUT1 and GLUT3 transporters, respectively (Rumsey and Levine, 1998; Lykkesfeldt, 2002). It was reported that DHA is rapidly recycled in erythrocytes predominantly via glutathione-dependent DHA reductases and with small contributions from reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent DHA reductases, such as thioredoxin reductase (Rumsey and Levine, 1998) (Figure 2-10).

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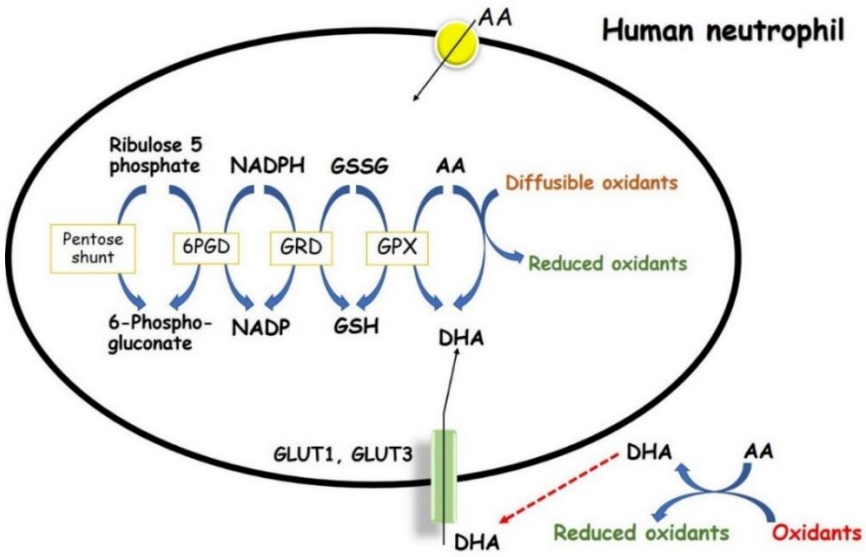


**Figure 2-10** The recycling of vitamin C in neutrophil of human; AA, ascorbic acid; DHA, dehydroascorbic acid; GSSG, oxidized glutathione; GSH, reduced glutathione; GPX, glutathione peroxidase; GRD, glutathione reductase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NADP, oxidized form of nicotinamide adenine dinucleotide phosphate; 6PGD; GLUT, glucose transporter

## X. Renal excretion of vitamin C

Vitamin C is freely filter at the glomerulus and reabsorbed at proximal tubule by active transport via sodium-dependent vitamin C transporter type 1 (SVCT1) (Martin et al., 1983; Rose, 1986). In human, the maximal reabsorption rates were relatively constant at 1.5 mg/100 ml glomerular filtrate (Oreopoulos et al., 1993).

## Y. Vitamin C, hyperoxaluria and CaOX nephrolithiasis

Oxalate or oxalic acid, the major metabolic waste in the body, can be generated from ascorbic acid metabolism (Lamarche et al., 2011). There are two major sources of oxalate, the exogenous oxalate from citrus fruit, some vegetable and endogenous oxalate from glyoxylate pathway (Marengo and Romani, 2008). Oral ingestion of ascorbic acid is not only increased vitamin C intestinal ingestion but also increased urinary excretion of oxalate (Baxmann et al., 2003). The CaOx stone forming is depended on the concentration ratio between urinary calcium to urinary oxalate (10:1 in the urine from normal people) (Marengo and Romani, 2008). Thus, an increase of urinary calcium to oxalate ratio might be the risk factor for CaOx stone formation. Patients with kidney impairment who ingested the mega-dose vitamin C (3-6.5 g/day) for one month were presented with acute oxalate nephropathy (Gurm et al., 2012). It is possible that ingestion of high dose vitamin C may increase the urinary oxalate excretion and increase the risk of CaOx stone (Lamarche et al., 2011). Nevertheless, there is no evidence that ingestion or intravenous injection of high dose vitamin C causes kidney stone formation. Nonetheless, it has not yet been fully investigated that an intravenous vitamin C injection could diminish CaOx kidney stone by antioxidant activity.

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## Z. The combination of vitamin E and vitamin C and the role of antioxidants

The combination of vitamin E and vitamin C might be a potent antioxidant effects due to the synergistic effect of both vitamins. Vitamin E, the hydrophobic vitamin, is found abundant in the cell membrane which is associated with the cell membrane NADPH oxidase subunits complex (Schramm et al., 2012). In addition, vitamin C is located in the cytoplasmic and mitochondrial compartments which are associated with superoxide dismutase (Castillo-Martin et al., 2014). When there are co-antioxidants, ascorbic acid helps vitamin E recycling mechanism in the cells (Rimbach et al., 2002) as shown in Figure 2-11.

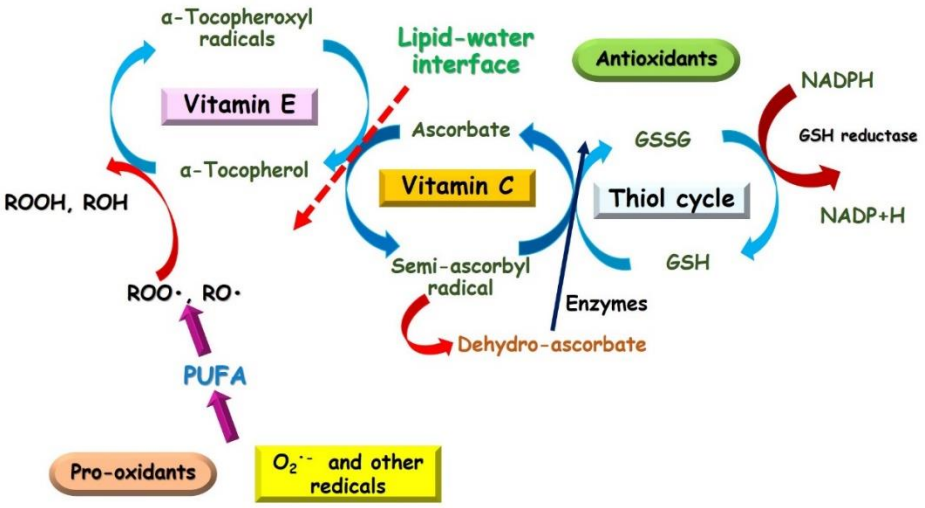


**Figure 2-11** The beneficial effects of vitamin C and vitamin E in combination. Vitamin C increases cellular vitamin E recycling.

## AA. The action of combination of vitamin E and vitamin C

The vitamin E and vitamin C are chain-breaking antioxidants. Hydrophobic cellular compartment, vitamin E plays a major role in free radical scavenger. However, there are many water-soluble chain-breakings in hydrophilic cellular compartment such as glutathione, cysteine, uric acid and vitamin C. Therefore, regeneration of vitamin E levels in general cell could be due to maintain its function and redox homeostasis in lipophilic compartment. Vitamin E interacts with vitamin C at the polar chromanoxyl head group of vitamin E which located near membrane water soluble border (Niki, 1985).

1. Cooperative inhibition of oxidation

Oxidation in water-soluble initiation condition, vitamin C inhibited the free radical and promoted a clear inhibition period before free radical attack the lipid bilayer. Vitamin E also suppressed free radical and promoted inhibition period. Combination of vitamin C and E promoted inhibition period which was similar to the summation of their separate inhibition periods.

2. Synergistic inhibition of oxidation

The synergistic effect of vitamin E and vitamin C appeared in condition of lipid-soluble oxidation. Free radicals were generated in cellular membrane. Administration of vitamin C separately could not induce inhibition of oxidation reaction while given

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vitamin E exerted oxidation inhibition effects. However, combination caused the higher inhibition effect than vitamin E alone.

Therefore, combination of vitamin E and C could be the most effective antioxidant supplement in imbalance redox homeostasis in many disease and preventive medicine.

## AB. Usage of combined vitamin E and vitamin C in kidney injury condition

In gentamicin induced nephrotoxicity rats, combination of vitamin E and vitamin C reduced LDH, NAG and ALP compared with gentamicin treated rats alone (Kadkhodaee et al., 2005). In gentamicin-treated Guinea pigs, SOD, GSH and CAT activities were lower significantly compared to control group and group receiving gentamicin plus vitamin C and vitamin E (Kavutcu et al., 1996). It has been hypothesized that gentamicin damaged renal tubular cell by attacking the mitochondria and increased ROS production (Wohlgemuth et al., 2014). Thus, the combination of vitamin E and vitamin C could help to support mitochondrial cellular stability and improve antioxidants status. In another renal toxic agent such as cisplatin, a platinum-based anti-cancer drug, it caused renal damage by apoptosis, inflammation, vascular ischemic reperfusion injury and over production of oxidative stress (Pabla and Dong, 2008). Ajith and colleagues (2009) found that the combination of vitamin C and vitamin E could reduce kidney lipid peroxidation, and improve renal GSH levels in mice

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with cisplatin induced nephrotoxicity. Moreover, in pyelonephritis rat model which were induced by inoculation of Escherichia coli after intramuscular injection of 10 mg/kg of gentamicin caused increase in plasma creatinine and renal scar formation. Giving vitamin C and E to pyelonephritis rat model could reduce plasma creatinine and renal scar formation (Emamghorashi et al., 2011). Thus, combination of vitamin C and vitamin E might be advantage in patients with many renal toxicity conditions.

Thamilselvan and colleagues (2014) studied in proximal tubular cell line (LLC-PK1) which injury was induced by oxalate at physiological concentrations. They reported that oxalate induce LLC-PK1 injury by increased ROS, increased cellular injury enzymes and reduced cellular antioxidants. The incubation of damage LLC-PK1 with vitamin E plus vitamin C could significantly improve cellular damage and antioxidant status compared with treated group and single administration of vitamin E or vitamin C group alone. Hence, the combination of vitamin E and vitamin C might have synergistic effects to prevent cellular damage from oxalate and CaOx stone. However, the *in vivo* study of combination effects of vitamin E and vitamin C with CaOx stone has not yet been elucidated.

## AC. Klotho protein: generation, structure, classification and distribution

In the recent year anti-aging protein, Klotho protein, has been widely studied in many disease conditions. The klotho gene was first identified in 1997 by Kuro-o and co-workers. It is expressed dominantly in distal convoluted tubules in the kidney and choroid plexus in the brain (Kuro-o et al., 1997). It was also detected in other endocrine organs such as pituitary, parathyroid, pancreas, ovary, testis and placenta (Kuro-o et al., 1997). The klotho gene-deficiency mouse developed premature multi-organ failure such as growth retardation, shorten life span, hyperphosphatemia, skin atrophy, loss of cognition. Thus, these data indicated that klotho may be antiaging gene (Kuro-o et al., 1997; Kurosu et al., 2005). Klotho protein is divided into three types, full length membrane, soluble and secreted form (Xu and Sun, 2015) (Figure 2-12).

The functions of different forms of Klotho protein were more revealed but not completely. The functions of full length membrane are co-receptor of fibroblast growth factor family 23 (FGF23) and fibroblast growth factor (FGF) which control active vitamin D formation and works on multifunctional homeostasis in renal handling of electrolytes including calcium and phosphate (Kurosu et al., 2006; Urakawa et al., 2006). The secreted and soluble forms are now discovered more than ten functions in physiological control of the body such as regulation of ion transporter (Ca and K), fibrogenesis, cancer metastasis, regulation of lipid raft formation (Dalton et al., 2017),

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regulation of cellular senescence by suppression of Wnt-beta catenin signaling pathway, cardioprotection and inhibition of insulin and insulin-like growth factor-1 (IGF-1).

It is a superior biomarkers of kidney injury because soluble Klotho protein reduced rapidly after kidney injury (Hu et al., 2010; Hu and Moe, 2012; Torregrosa et al., 2015). The study revealed that in CKD and AKI rat's model, naked plasmid encoding secreted klotho (pV5-sKlotho) IV injection could protected injury lesions in the kidney tissue (Zhou et al., 2015). Moreover, the same research group found that fibrosis in kidney tissues were reduced in pV5-sKlotho injection group. It is implied that Klotho protein could modulate kidney injury and reverse many diminished functions after kidney disease occur. Moreover, report of the single nucleotide polymorphism of Klotho gene in CaOx stone patients at the positon G395A was found (Telci et al., 2011). Although the association between Klotho protein and calcium oxalate stone is incompletely explained.

Recently, the levels of urinary Klotho protein in human CKD patients were significantly correlated with the estimated GFR (eGFR) and severity of CKD stage (Akimoto et al., 2012; Kim et al., 2015). Thus, Klotho protein levels can be used as an early biomarker for detecting the alteration and progression in CKD patients.

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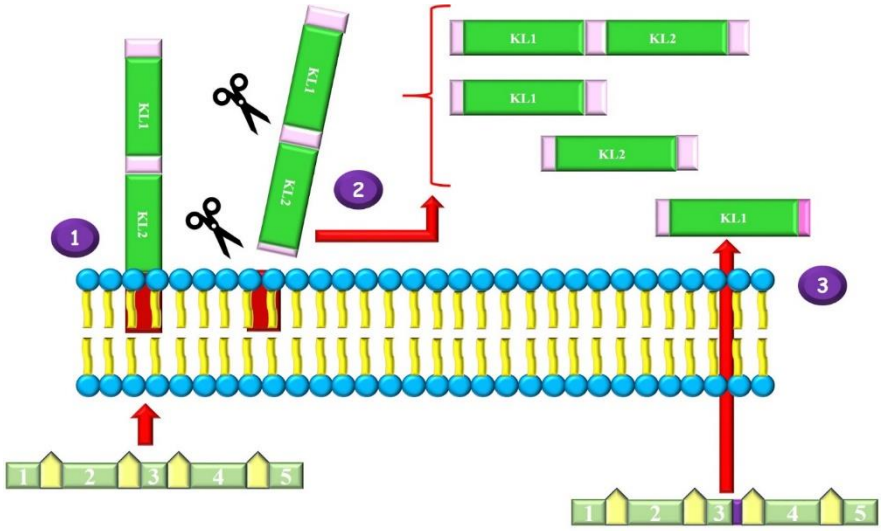


**Figure 2-12** The three types of Klotho protein: 1. full length trans-membrane protein, 2. circulation or soluble form (molecular weight 130 kDa) and 3. secreted form. The full length trans-membrane form was cutting by a disintegrin and metalloproteinase domain- containing protein family 10 and 17 (ADAM10, ADAM17).

## AD. Renal handling of soluble Klotho

The first study which demonstrated the renal handling of soluble form of Klotho protein in anephric rats and the direction of Klotho protein transported in opossum kidney cell line were performed (Hu et al., 2016). The highest expression of Klotho gene and Klotho protein in the kidney is the main source of soluble Klotho protein which cleared completely via the kidney. The half-life of endogenous soluble Klotho is 26.6 hours. Moreover, the direction of transcytosis and trafficking of Klotho protein in proximal tubule is from basolateral side to apical membrane only.

## AE. Klothoprotein and oxidative stress

It has been reported that Klotho protein could be used as preventive molecule for renal impairment (Hu et al., 2016). Mouse transfected with Klotho gene decreased intracellular superoxide concentration through cyclic adenosine monophosphate (cAMP) (Wang et al., 2012). However, the association between Klotho protein levels and redox regulation molecules such as SOD, catalase and glutathione in calcium oxalate stone have not yet been fully investigated.

## AF. Klotho protein, hyperoxaluria and CaOx nephrolithiasis

It is documented that the membrane form of Klotho protein plays a central role in the pathogenesis of CaOx urolithiasis due to its association with vitamin D-calcium-phosphorus regulation (Kuro, 2011). However, the effect of the soluble form of Klotho protein has not yet been investigated in kidney. Importantly, the soluble Klotho protein directly controlled the TRPV5 (the apical transient receptor potential vanilloid $Ca^{2+}$channel) and TRPV6 which were the calcium channel in the kidney and intestine, respectively owning to its glucuronidase enzyme activity (Huang, 2010). The soluble Klotho protein cleaved the N-glycan structure in the TRPV5 resulting in the inhibition of internalization of calcium channel, thus enhanced reabsorption of calcium from the ultrafiltrate at distal convoluted tubule (Cha et al., 2009). Telci et al. (2011) found that there are significant differences in single nucleotide polymorphisms (SNPs) between healthy people and kidney stone formation. Therefore, Klotho protein might

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play an important role in CaOx kidney stone formers and could be used as a future diagnostic and therapeutic marker in patients with CaOx stone. However, the study of Klotho protein concentration and function in hyperoxaluric rats has not yet been thoroughly studied.

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# CHAPTER III

# MATERIALS AND METHODS

The study was divided into two parts as follow:

**Part I**. Protective effects of vitamin E and vitamin C supplement on renal functions, oxidative stress and renal handling of organic and inorganic substances in HLP-induced hyperoxaluric rats.

**Part II**. Effects of vitamin E and vitamin C supplement on oxidative stress, Klotho protein levels and Klotho protein mRNA expression in HLP-induced hyperoxaluric rats.

## 1. Approvals

This experiment was approved and conformed by Chulalongkorn University Care and Use Protocol (CU-ACUP), Faculty of Veterinary Science, Chulalongkorn University in protocol review number 1431056.

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## 2. Animal managements and experimental designs

The seventy Male Sprague Dawley rats obtained from National Laboratory Animal Center, Mahidol University (NLAC), weighed between 250-350 grams were included. All rats were housed in the 12:12 hours dark and light cycle and permitted for access free water and food ad libitum. All animals were acclimatized to the environment for 7 days before experiment. The experimental rats were divided randomly into 5 groups as follows:

**Group 1,** control group, rats were received regular drinking water. An intraperitoneal injection of olive oil was performed (0.2 ml/100g body weight) as a placebo of vitamin E (Vit E) and an intravenous injection of isotonic saline was administered (0.2 ml/100g body weight) as a placebo of vitamin C (Vit C).

**Group 2,** hyperoxaluric group, rats were received 2% HLP in drinking water throughout the experimental period. An intraperitoneal injection of olive oil (0.2 ml/100g body weight) and an intravenous injection of isotonic saline (0.2 ml/100g body weight) were performed as a vehicle of Vit E and Vit C, respectively.

**Group 3,** hyperoxaluric rats with vitamin E supplement group, in addition to hyperoxaluric group, Intraperitoneal injection of Vit E (200 mg/kg body weight) and an intravenous injection of isotonic saline (0.2 ml/100g body weight) were performed.

**Group 4,** hyperoxaluric rats with Vit C supplement group, in addition to hyperoxaluric group, intraperitoneal injection of olive oil (0.2 ml/100g body weight) and intravenous injection of Vit C (500 mg/kg body weight) were performed.

**Group 5,** hyperoxaluric rats with vitamin E and vitamin C supplement group, in addition to hyperoxaluric group, rats were received an intraperitoneal injection of Vit E (200 mg/kg body weight) and an intravenous injection of Vit C (500 mg/kg body weight).

The route, duration and dosage of Vit E were followed by the study of Huang and co-workers (2006) which used 200 mg/kg body weight of vitamin E in EG-induced hyperoxaluria prevented CaOx crystal formation. Additionally, the route, duration and

dosage of Vit C were followed by the study of Korkmaz and Kolankaya (2009) and Robitaille et al. (2009) due to the protective effects of kidney injury from gentamicin-induced nephropathy and the levels of oxalate excretion, respectively.

The number of the experimental rats in each group for study in part I and part II were shown in Table 3.1

**Table 3.1** The randomization of seventy experimental rats into five groups for the study in part I and part II.

| Experimental groups | Study part I (n=37) | Study part II (n=33) |
|---|---|---|
| Group 1 | 8 | 7 |
| Group 2 | 8 | 7 |
| Group 3 | 7 | 6 |
| Group 4 | 7 | 6 |
| Group 5 | 7 | 7 |

## 3. Drugs, infusion solutions and chemical reagents

3.1 Vitamin E (d-alpha-tocopherol) (DURVET, INC, Blue Springs, MO, USA) had a concentration 1 I.U. of d-alpha-tocopherol = 0.67 mg of natural form. The 300 I.U. per ml (201 mg/ml) of vitamin E was used in dosage 200 mg/kg intraperitoneally injection.

3.2 Vitamin C (Ascorbic acid, 500 mg / 2 ml) (Atlantic Laboratories Corporation Ltd., Bangkok, Thailand) (250 mg/ml). The ascorbic acid 500 mg/kg body weight was intravenously injection.

3.3 trans-4-Hydroxy-L-proline (HLP) (ACROS ORGANICS, New Jersey, USA). The 2% w/v HLP added in drinking water throughout the experimental period.

3.4 Pentobarbital sodium (Nembutal®, 5.47g per 100 ml) (CEVA Sante' Animale, Libourne, France). The pentobarbital sodium was used for anesthetic drugs by intraperitoneally injection in a dosage 60 mg/kg BW.

3.5 0.9% Normal saline was used as placebo for vitamin C by intravenous injection and vehicle for clearance study.

3.6 Olive oil (100% purification, VIDHYASOM Co, Ltd., Bangkok) was used as a placebo for vitamin E by intraperitoneal injection.

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3.7 Inulin (Sigma aldrich, 25g) (SIGMA-ALDRICH, Co, MO, USA) was prepared to 1% solution for renal clearance study.

3.8 PAH (Sigma aldrich, 500g) (SIGMA-ALDRICH, Co, MO, USA) was prepared to 0.2% solution for renal clearance study.

3.9 Lithium Chloride solution was prepared to 0.07% and 0.015% for lithium clearance study.

3.10 Flushing solution was containing of 0.1M sodium phosphate buffer.

3.11 Fixation solution (4% paraformaldehyde) was prepared from the combination between 0.2 M sodium phosphate buffer and 8% of paraformaldehyde.

## 4. Experimental protocol

### 4.1 Study part I

All rats were subjected to experimental study for 21 days as previous described in HLP induced hyperoxaluric rat model by Khan et al. (2006) and Zuo et al. (2011). The experimental rats were studied in renal hemodynamics on day 21 (Figure 3-1). The data of body weight, food and water intake was recorded daily. After acclimatization periods, all rats in each group were enrolled into the individual metabolic cage for 24 hrs urine collection for measurement of substances and volume (U0). The study in metabolic cage was repeated on day 10 and 20 (U10 and U20) (Figure 3-1).

One milliliter of urine sample from metabolic cage was obtained for urinalysis and measurement of urinary protein and creatinine concentration. The rest of the urine was stored at -20oC for further analysis of the concentrations of the electrolytes (Na, K, Cl, Ca, Mg and Zn), and stored at -70°C for analysis of osmolarity, oxalate, citrate, MDA, and TAS.

The indirect blood pressure (BP1 and BP11) was performed on day 1 and day 11 after animals were released from metabolic cage. Each rat was anesthetized using 60 mg/kg pentobarbital sodium injected intravenously while 0.5 ml of blood sample was collected by trimming the tail vein for measurements of hematocrit, and the concentrations of blood urea nitrogen (BUN) and creatinine (Figure 3-1). The 2% HLP

(w/v) were added into drinking water for rats in group 2-5. Vit E (200mg/kg body weight) was injected intraperitoneally on day 1, 6, 11 and 16 in group 3 and 5 while olive oil was used as a placebo of vitamin E in group 1, 2 and 4. Dosage, route and duration of Vit E injection were followed as studied by Huang et al. (2006). Vitamin C was injected intravenously on day 1 and 11 in group 4 and 5 while the 0.9 % NSS was administered as a placebo of vitamin C in group 1, 2 and 3. Based on the study from Korkmaz and Kolankaya (2009) and Robitaille et al. (2009), 500 mg/kg body weight of Vit C was considered as optimum dosage and used in the present study.

Five ml of blood were obtained from cardiac puncture for the measurements of hematocrit (Hct), BUN, creatinine (Cr), electrolytes (Ca, Mg and Zn), osmolarity and MDA on day 21. After the rats were released from metabolic cage on day 21, the renal clearance study was accomplished for evaluation of renal functions and renal hemodynamic parameters (Figure 3-2).

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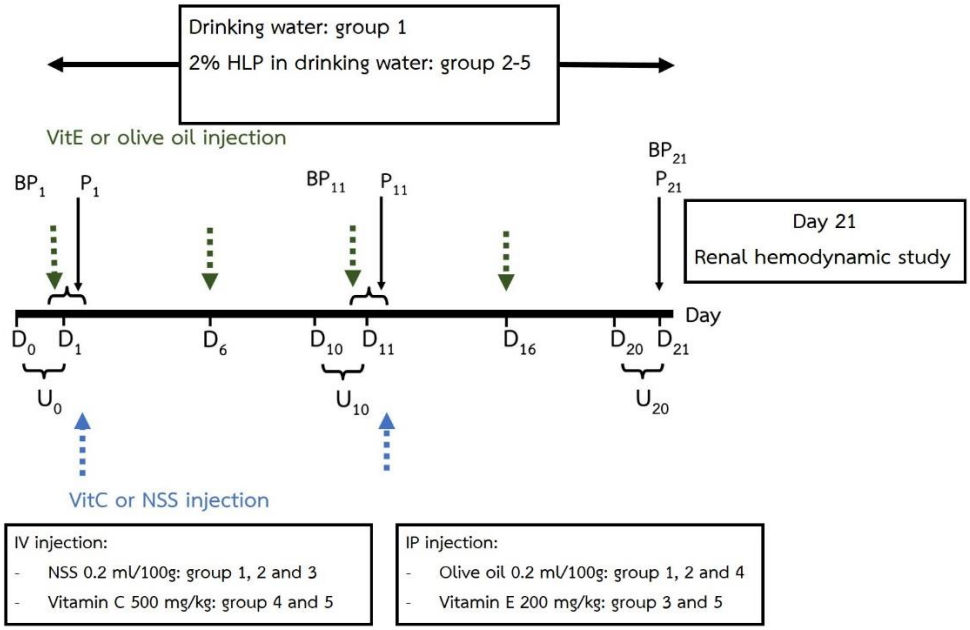


**Figure 3-1** Experimental procedure for vitamin E and vitamin C injection, renal excretion of substances and the determination of parameters in urine and plasma.

P1 and P11: Hematocrit, BUN and creatinine levels in plasma on day 1 and day 11

P21: Hct, BUN, creatinine, electrolytes (Na, K, Cl, Ca, Mg and Zn), osmolarity, malondialdehyde (PMDA), oxalate and citrate levels in plasma on day 21

BP1, BP11: Indirect blood pressure on day 1 and 11

BP21: Direct blood pressure on day 21

U0, U10 and U20: urinalysis, UPC ratio, creatinine, electrolytes (Na, K, Cl, Ca and Mg), osmolarity, oxalate, citrate, MDA, TAS and Klotho protein levels in urine on day 0, 10 and 20

Day 21: Renal clearance study (clearance of inulin ($C_{In}$), clearance of para-aminohippurate ($C_{PAH}$) and clearance of lithium ($C_{Li}$)).

### 4.1.1 Experimental protocol for renal hemodynamic study

On day 21, renal hemodynamics, tubular handling of Na and water were evaluated by the clearances of inulin, para-aminohippurate (PAH; $C_{PAH}$) and lithium

($C_{Li}$), respectively. The experimental rats were anesthetized by used an intraperitoneal injection of pentobarbital sodium at dosage 60 mg/kg BW. The trachea was approached then by ventral midline incision at the neck position. The polyethylene tube (PE 240) was inserted directly into the trachea of the rats and ligated with the suture for prevention of tracheal obstruction. After that, the carotid sheet which lied next to the trachea was blunted by curved mosquito forceps and then the internal carotid artery was stabbed and subsequently placed the PE-50 which connected to physiograph (BIOPAC® Systems, Inc, Goleta, CA, USA) while data acquisition was performed with specific software (AcqKnowledge®, BIOPAC® Systems, Inc, Goleta, CA, USA). Then, the stage of surgical anesthesia was maintained at stage III. After that, the incision of the ventral abdominal midline was achieved for approach, stab the urinary bladder and place the PE 240 into the bladder for collection of the urine. Afterward, the inguinal area of the right leg was approached to locate and dissected the femoral artery and vein. The PE-50 was placed directly into both femoral vessels for infusion the solution and collection of blood (Figure 3-2).

During 45 minutes of equilibration periods, the mixture of solution containing 1% inulin, 0.2% PAH and 6% mannitol was infused at the rate of 1 ml/hr per 100 g body weight into the femoral vein. After equilibration period, the 0.20 ml of 0.07 M lithium chloride (LiCl) solution was given as a bolus and followed by continuous infusion of the same mixture containing inulin, PAH and mannitol with addition of 0.015 M of LiCl solution, which was kept at the same rate throughout the study (Figure 3-2).

Ten minutes after 0.015 M of LiCl administration, three consecutive urine collections were performed every 20 minutes for measurements of urine volume and concentrations of lithium, inulin and PAH. The 0.5 ml of blood were collected at the midpoint of each urine collection to determine the concentrations of lithium, inulin and PAH. After each blood collection, 6% bovine serum albumin was administered at an equal volume to replace blood loss.

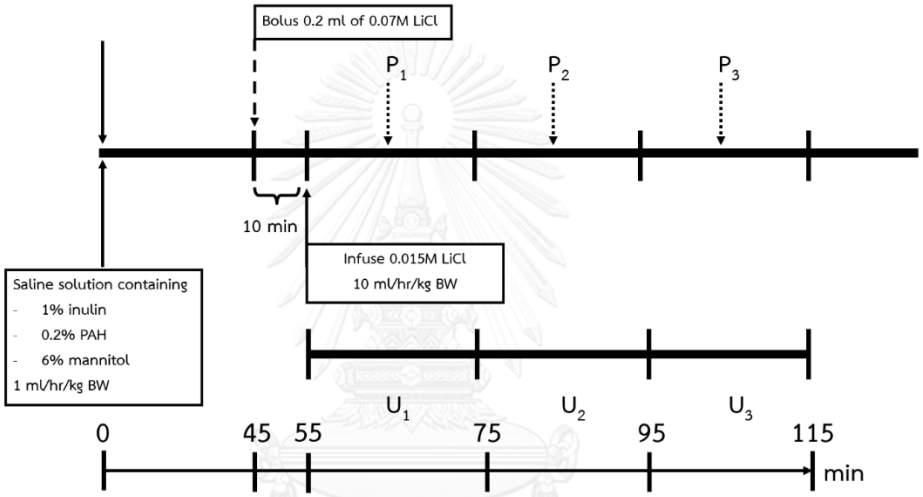


**Figure 3-2** Experimental protocol for renal clearance study. The saline solution containing 1% inulin, 0.2% PAH and 6% mannitol was infused in the rate 1 ml/hr/kg BW until equilibration period at 45 min. After that, 0.07M LiCl was given in bolus 0.2ml followed by 0.015M LiCl infusion (10ml/hr/kg BW). The three-consecutive urine and blood were collected every 20 min for further determination of concentration of substances in urine and plasma.

**4.2 Study part II**

The thirty-three rats were enrolled to experimental study for 21 days and randomized into five groups of experiment as described in study. part I. The daily data of the body weight, food and water intake were recorded in all rats. The 24 hrs urine in each rat was collected by placing a rat in individual metabolic cage on day 0 and day 20 (Figure 3-3). One milliliter of urine was used for urinalysis and determination of urinary protein excretion and creatinine concentration. The rest of the urine was kept at -20°C for further analysis of the electrolytes level (Na, K, Cl, Ca, Mg and Zn), and stored at -70°C for measurement of osmolarity, oxalate, citrate, MDA, and TAS.

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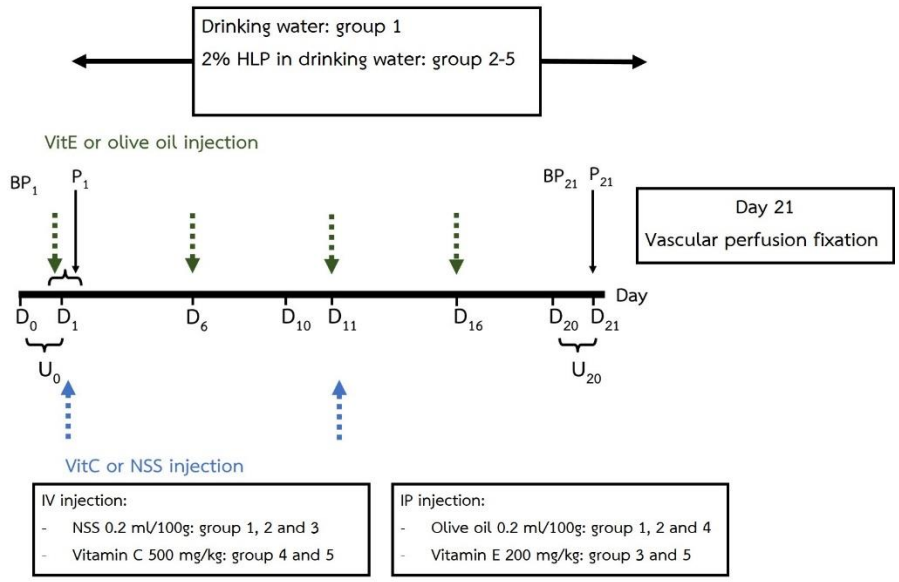


**Figure 3-3** The experimental protocol for study of Klotho protein in hyperoxaluric rats.

P1 and P21: Hematocrit, BUN and creatinine

P21: Hematocrit, BUN, creatinine, electrolytes (Na, K, Cl, Ca, Mg and Zn), osmolarity, MDA, oxalate, citrate and Klotho protein

BP1: Indirect blood pressure at day 1

BP21: Direct blood pressure at day 21

U0, and U20: urinalysis, UPC ratio, creatinine, electrolytes ($Na^+$, $K^+$, $Cl^-$, $Ca^{2+}$ and $Mg^{2+}$), osmolarity, oxalate, citrate, MDA, TAS and Klotho protein

Vascular perfusion fixation: kidney Klotho protein, histopathology and kidney oxidative status

**4.2.1 Experimental protocol for kidney Klotho protein and kidney oxidative stress**

After the rats were released from metabolic cage on day 21, all rats were anesthetized by used intraperitoneal injection of pentobarbital sodium in dose 60 mg/kg BW. The trachea and carotid sheet were approached by ventral midline incision of the neck. The tracheostomy was performed for the prevention of tracheal obstruction by inserted directly of polyethylene catheter (PE 240). The insertion of PE 50 into the internal carotid artery was achieved for measurement of direct blood pressure and collection of blood sample. One milliliter of blood sample was used for determination of Hct, BUN and Cr while the rest of plasma was stored at -70°C for further analysis of electrolytes (Na, K, Cl, Ca, Mg and Zn), osmolarity, Klotho protein and MDA.

After that, the ventral midline abdominal incision was approached to locate the kidney, abdominal aorta and vein, renal artery and renal vein. The right kidney was ligated and removed immediately and washed with ice-cold Na-PBS before kept at -70°C for further examination of oxidative stress parameters (catalase (CAT), superoxide dismutase (SOD), reduced form of glutathione (GSH), MDA and mRNA expression of Klotho protein.

Consequently, the vascular perfusion fixation was performed via abdominal aorta and vein. The left kidney was flushed with ice-cold Na-PBS pH 7.4 and subsequently fixed by used a 4% paraformaldehyde for 5 minutes. Then, left kidney

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was removed and fixed in the 0.1M Na-PBS containing 4% w/v paraformaldehyde overnight at 4°C for further study of immunohistochemistry and histopathology investigation. After the study, all rats were euthanized using over dosage of pentobarbital sodium.

## 5. Analytical procedures for determination of urine, plasma and kidney tissue samples

The levels of of PCr, BUN and UCr were determined using colorimetric method by automated analyzer (The IL ILab 650 Chemistry Analyzer, Diamond diagnostic, MA, USA). The flame photometer (Frame photometer 410C, Ciba Corning Inc., USA) was used for determination of plasma and urine electrolytes concentrations (Na and K) while chloridometer (Chloride analyzer 925, Ciba Corning Inc., USA) was used for measurement of plasma and urine chloride levels. The osmometer (Advanced Instrument Inc., USA) was used for evaluation of plasma and urine osmolarity. The concentrations of inulin in urine and plasma were measured by using the Anthrone method (Young and Raisz, 1952) while the concentration of PAH in urine and plasma were determined by using Brun method (Brun, 1951). The concentrations of plasma and urine Ca, Mg and Zn were assessed using inductively coupled plasma optical emission spectrometry (Perkin Elmer™ Optima 5400, Waltham, MA, USA) while the levels of oxalate and citrate were measured using capillary electrophoresis (P/ACE™ MDQ CE Beckman Coulter, Fullerton, CA, USA). Urinary TAS was investigated by using

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the method of Chrzczanowicz et al. (2008) while the concentrations of MDA in urine, plasma and kidney tissues were evaluated by using the method of Ohkawa et al. (1979). The levels of reduced GSH in the kidney tissues were measured using the method of Beutler et al. (1963). The activity of SOD and catalase was evaluated using the method of McCord and Fridovich (1969) and the method of Aebi et al. (1968), respectively. The concentrations of protein in urine and kidney tissues were measured by Lowry's method (Lowry et al., 1951).

## 6. Urinalysis study

The specific gravity of urine was assessed by refractometer from 0.5 ml of urine in each rat while the pH and the chemical properties of urine samples were measured using the urine strip test (Combur9 Test®, Roche Diagnostics GmbH, Mannheim, Germany). After that, the 0.5 ml of urine was centrifuged at 2500 rpm for 10 minutes and discarded the supernatant. Then, the sediment of urine was stained with Sternheimer-Malbin and subjected to microscopic visualization for evaluation of calcium oxalate crystal. The CaOx crystal was quantified by examining the sections at x40 with the average from five fields per section. The average crystal count was recorded as 0: no crystal deposit per field, 1: 1-2 crystal deposits per field, 2: 3-5 crystal deposits per field, 3: 6-10 crystal deposits per field and 4: >10 crystal deposits per field.

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## 7. Determination of urine, plasma and kidney MDA

Malondialdehyde (MDA) was determined using a method of Ohkawa (Ohkawa et al., 1979). The MDA was measured in the form of thiobarbituric acid reacting substances (TBARS). The 500 µl of urine or plasma was added in a reaction mixture of 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid and 6.6 ml of water. After mixing the mixture vigorously, the mixture was heated at 95oC for 1 hour. Afterward, the mixture was cooled with tap water then n-butanol and pyridine solution (15:1, v/v) were added and shaken strongly for 1 minute before centrifugation at 5000 rpm for 10 minutes. The supernatant layer which containing the lipid peroxidation product, organic layer, was measured the absorbance at 532 nm by spectrophotometric method and the value of MDA in urine or plasma were expressed as nmol/ml.

To evaluate the product of lipid oxidation, MDA, in the kidney tissue, the renal cortex was homogenized in reagent that compose of 1.15% potassium chloride and 0.003 M EDTA (1:10 w/v). The homogenate was centrifuged at 600 g for 15 minutes. Then, the 0.4 ml of supernatant was added into the reaction solution as described for MDA determination in urine and plasma samples. MDA value was expressed as nmol per milligram kidney protein which is determined by Lowry method.

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## 8. Determination of urine TAS

Total antioxidant status in urine was evaluated by used the method of Chrzczanowicz et al. (2008). The principle of the assay is the interaction between a stable free radical a, a-Diphenyl-b-picrylhydrazyl (DPPH) and antioxidant enzymes in the samples. Adding 20 µl of samples to the mixture of 400 µl sodium phosphate buffer plus 400 µl DPPH, then mixed carefully and incubated at the room temperature for 20 min. The absorbance was reccorded at 520 nm. The radical scavenging activity (% inhibition) was presented as percentage of DPPH radical elimination which calculated by the following formula; % inhibition= [(ODblank- ODtest)/ ODblank] × 100.

## 9. Determination of urine and plasma Li, Ca, Mg and Zn concentration

The levels of Ca, Mg and Zn in urine and plasma samples were determined using inductively coupled plasma optical emission spectrometry (ICP-OES) method. The system of ICP-OES was composed of pump, nebulizer, spray chamber, light source unit, radiofrequency unit, spectrophotometer and a detector connected with a data processing unit.

The principle of ICP-OES was the optical emission spectrometry. After the sample was pumped into the nebulizer and generated the mixture of gases of sample and inert gas (argon), the atoms of mixtures were excited. After that, the excited-atoms were flowed into the low energy site and the spectrum rays were then released and emitted wavelength corresponding with a specific pattern in each inorganic metal. The

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intensity of each elements was detected and reported in value of part per million (ppm) which standardized with standard curve.

## 10. Determination of urine and plasma oxalate and citrate concentrations

The capillary electrophoresis (CE) was used for determination of the concentrations of oxalate and citrate in urine and plasma samples. The specification of CE is to separate the difference substances by its size and polarity under the electrical field along with capillary tube. Separation and quantification of oxalate and citrate using CE at the optimal length of capillary tube and buffer were based upon the study by Garcia and co-workers (2001). The reverse-polarity of CE with an applied voltage of -15kV at 25oC were introduced for detection of oxalate and citrate levels in plasma and urine samples. An optimal buffer, pH and background electrolyte contained borate buffer (40 mM), phosphate buffer (100 mM) and tetradecyltrimethyl ammonium bromide (TTAB, 0.5 mM) while the wavelength was detected at 195 nm.

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## 11. Determination of urine protein concentrations

The concentrations of protein in urine was measured by used the method of Lowry (1951).

## 12. Determination of urine and plasma Klotho protein concentrations

The soluble form of Klotho protein was investigated by a sandwich ELISA assay (SEH757Ra, Cloud-Clone Corp, Houston, TX, USA). The urine and plasma were

incubated with pre-coated microplate of biotin-conjugated antibody specific to Klotho protein. Afterward, avidin conjugated to Horseradish Peroxidase was added and incubated to each well of microplate. Substrate solution was added subsequently and exhibited a change in color of biotin-conjugated antibody and enzyme-conjugated Avidin-substrate complex. The stop solution, sulphuric acid, was added for termination the reaction. The color was measured at wavelength 450 nm. The Klotho protein levels in the samples were determined by compared with the O.D. of standard curve.

## 13. Determination of kidney reduced GSH concentrations

Kidney GSH was evaluated using a method of Beutler (1963). Renal cortex was suspended in 1.8 ml of 100 ml mM KCl and 0.003 M EDTA and homogenized as described above for determination of MDA. The homogenates were centrifuged at 600 g for 15 minutes, added 1 ml of supernatant to 1.5 ml of metaphospholic acid and centrifugated at 3000 g for 10 minutes. Reduced GSH was measured by adding 500 µl of supernatant to 2.0 ml of 0.2 M phosphate buffer and 0.25 ml of 0.04% 5,5 dithiobis 2-nitrobenzoic acid. The absorbance was read at 412 nm against standard GSH. The kidney GSH was expressed as nanomoles of GSH per milligram of kidney protein.

## 14. Determination of kidney catalase activity

Kidney catalase activity was determined using a method of Aebi et al. (1968) and expressed as sec-1 per milligram homogenate protein. Slice of renal cortex tissue (0.2 g) was homogenized with 1% Triton X-100. One hundred microliters of supernatant

were added in a quartz cuvette containing 1.9 ml phosphate buffer and then add 1 ml of 30 mM $H_2O_2$ to start the reaction. The change in absorbance was detected at 240 nm every 30 secs for 1-2 min using UV-VIS spectrophotometer. The external standard catalase was used and the activity of catalase was expressed as unit per milligram kidney protein which determined by Bradford method.

## 15. Determination of kidney SOD activity

The kidney super oxide dismutase (SOD) activity was assessed by SOD assay as previous described by McCord and Fridovich (1969). The renal cortex was suspended in 1.5 ml of PBS buffer and homogenized. The SOD activity expressed in unit which inhibits the rate of cytochrome c reduction by 50%. The data was related to standard preparation utilizing a plot $1/\Delta E\ min^{-1}$ versus standard. The solution A is 0.76 mg of xanthine and 24.8 mg of cytochrome c was mixed with 100 ml of 50 mM phosphate buffer and kept at 25 °C. The solution B was xanthine oxidase in 0.1 mM EDTA which was stored on ice. Adding 2.9 ml of solution A in cuvette and then followed by 50 µl of SOD standard or sample. After that, 50 µl of solution B was added when the reaction was started the absorbance was recorded at 550 nm and plotted using $1/\Delta E\ min^{-1}$ derived from the linear part of the reaction versus concentration of SOD standard.

## 16. Procedure for kidney histopathology investigation

The kidneys were fixed with 4% paraformaldehyde in 0.1 mol/l PBS for 16 h at 4 °C and then embedded in paraffin. Paraffin sections (3 µm thick) are deparaffinized

and hydrated, and the sections are stained with hematoxylin and eosin to examine histological changes in the kidney. Histopathological lesions both in cortex and medulla of glomerulus and tubular compartment were examined under a light microscope by veterinary pathologist using high-power fields (HPF) (X40, magnification). The glomerular lesions including mesangial cells hypertrophy, leukocyte accumulation, Bowman's capsule space dilation and crystal deposition were noted from the average of 50 glomeruli (Weening et al., 2004). Microscopic lesions of tubular compartment including tubular dilatation, tubular cell flattening, tubular cell vacuolization and deposition of tubular cast were noted. Ten HPF areas in each slide were randomly selected and scored according to 0=NRL (no remarkable lesion); 1=mild, 25-50% area per HPF; 2=moderate, 51-75% area per HPF; 3=severe, >75% area per HPF (Weidemann et al., 2008).

## 17. Procedure for kidney Klotho protein expression evaluation

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Paraffin embedded of kidney tissue was deparaffinized and hydrated. All Kidney tissue slides were pretreated with citrate buffer (pH 6.0) and heat for 20 minutes. The 3% hydrogen peroxide was used for endogenous peroxidase block. Non-specific bindings were blocked by 45 minutes' incubation with 3% bovine serum albumin (BSA). Sections were incubated with rabbit anti-Klotho primary antibody (5 µg/ml, Abcam ab154163, Cambridge, MA, USA) at 4 °C overnight. Detection step was used EnVision® System HRP labelled polymer anti- rabbit (Dako, Glostrup, Denmark) at room temperature for 45 minutes followed by 3,3' diaminobenzidine chromogen (DAB

solution, Dako, Glostrup, Denmark). All sections were counterstained with Mayer's hematoxylin and examined under a light microscope. The positive area of Klotho protein was evaluated by using the iSolution program.

## 18. Procedure for kidney Klotho mRNA expression evaluation

The Klotho mRNA expression was determined by using real-time PCR as previously described (Freel and Hatch, 2012; Joshi et al., 2012; Frick et al., 2013). In brief, total RNA of Klotho protein in the kidney were extracted with RNeasy® Mini Kit (QIAGEN®, Hilden, Germany. The RNA purification was conducted according to manufacturer's instructions. The concentration of total RNA was determined by NANODROP 2000c spectrophotometer (Thermo Fisher SCIENTIFIC, Ma, USA). First-strand cDNA was synthesized from 100 ng/µl of RNA with Thermo Scientific RevertAid Reverse Transcriptase (Lot 00317047). The QPCR Green Master Mix HRox, 2x (biotechrabbit, Hennigsdorf, Germany) was used for quantitative real-time PCR master mix. The qPCR products analyzed by StepOnePlus™ Real-Time PCR System (AB Applied Biosystems, CA, USA).

The conditions used in the present study of both GAPDH and Klotho mRNA expression were shown in Table 3.2

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**Table 3. 2** The condition using in realtime PCR processes

| **GAPDH** | | | **Klotho** | | |
|---|---|---|---|---|---|
| step | temperature (°C) | duration | step | temperature (°C) | duration |
| Initial step | 95 | 2 min | Initial step | 95 | 2 min |
| 1.Denaturation | 95 | 15 sec | 1.Denaturation | 95 | 15 sec |
| 2.Annealing | 55 | 30 sec | 2.Annealing | 62 | 30 sec |
| 3.Extension | 72 | 15 sec | 3.Extension | 72 | 15 sec |
| 4.Detection | 79 | 40 sec | 4.Detection | 80 | 40 sec |
| Detection cycle = 40 cycles | | | Detection cycle = 45 cycles | | |

Gene activity was normalized to the average concentration of housekeeping gene (GAPDH). The estimated-length of Klotho and GAPDH was 120 base pairs and 350 base pairs, respectively. Primer sequences were described below (Freel and Hatch, 2012; Frick et al., 2013; Joshi et al., 2015).

Rat Klotho

Forward 5’-CGTGAATGAGGCTCTGAAAGC-3’

Reverse 5’-GAGCGGTCACTAAGCGAATACG-3’

Rat GAPDH

Forward 5’- TCCCTCAAGATTGTCAGCAA-3’

Reverse 5’- AGATCCACAACGGATACATT-3’

The relative change of klotho mRNA expression was calculated by comparative $C_T$ method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008).

## 19. Calculation

19.1 Mean arterial blood pressure = DP + 1/3(SP-DP)

19.2 GFR was calculated using inulin clearance ($C_{In}$) (µl/g/min) = ($U_{In}$ * $V_{In}$) / $P_{In}$

19.3 ERPF was calculated using PAH clearance ($C_{PAH}$)(µl/g/min) = ($U_{PAH}$ * $V_{PAH}$) / $P_{PAH}$

19.4 ERBF (µl/g/min) = (ERPF * 100) / (100-Hct)

19.5 Filtration fraction (FF) (%) = (GFR * 100) / ERPF

19.6 RVR (mmHg/µl.g-1.min-1) = MAP / ERBF

19.7 Filter load of substances (mg/min) = GFR x P

19.8 Urinary excretion of substances (mg/min) = U x V

19.9 Tubular reabsorption of substances = (GFR x P) – (U x V)

19.10 Osmolar clearance ($C_{Osm}$) = ($U_{Osm}$ x V) / $P_{Osm}$

19.11 Free water clearance ($C_{H2O}$) = V - $C_{Osm}$

19.12 Fractional excretion of substances = ((Us x V) / (Ps x GFR)) x 100

19.13 Clearance of substance = UV / P

19.14 Delivery of sodium out of proximal tubule (µl/g/min) = (ULi * VLi) / PLi

19.15 Reabsorption of water in proximal tubule (µl/g/min) = CIn - CLi

19.16 Reabsorption of sodium in proximal tubule (µEq/g/min) = (CIn - CLi) x PNa

19.17 Reabsorption of water in distal tubule (µl/g/min) = CLi – V

19.18 Reabsorption of sodium in distal tubule (µEq/g/min) = (CLi - CNa) x PNa

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19.19 The fractional excretion of sodium from proximal tubule ($FE_{Li}$) = CLi / CIn

19.20 Urinary excretion of sodium from proximal tubule = $C_{Na}$ / $C_{Li}$

19.21 Urinary excretion of water from proximal tubule = V / $C_{Li}$

19.22 Tiselius index =

$$\frac{4067 * Calcium^{0.93} * Oxalate^{0.96}}{(Citrate + 0.015) * Magnesium^{0.55} * V^{0.99}}$$

## 20. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The data obtained in between groups were compared with group 1 or group 2 using One-way ANOVA and One-way ANOVA on rank. The data obtained from the same group at different time points were compared using One-way repeated measured ANOVA or One-way repeated measured ANOVA on rank. The pairwise comparisons were performed using Dunn's method. The relationships between parameters were performed using Pearson's correlation and linear regression analysis. The *p*-value less than 0.05 was considered as significant difference.

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# CHAPTER IV

# RESULTS

## 1. Study part I

Protective effects of vitamin E and vitamin C supplement on renal functions, oxidative stress status and renal handling of organics and inorganics substances in HLP induced hyperoxaluric rats.

### A. General parameters (BW, food intake, water intake and blood chemistry)

*Body weight*

On day 21, the averages of body weight in group 1, 2, 3, 4 and 5 were 378.2±7.4, 397.9±15.9, 365.1±15.4, 407.9±10.7 and 370.6±14.6 grams, respectively. There was no significant difference among groups.

*Food and water intake*

Before experiment, food intake was only significantly lower in group 5 than group 1 within normal range, while the rest were indifferent. At the end of experiment, there was significantly lower in daily food intake in group 3 when compared with control rats (group 1-5; 23.31±0.795, 21.51±0.786, 18.34±2.151, 22.74±0.698 and 20.93±1.542 grams). The water intake was significantly increased in HLP group at day

10 of experiment compared with group 3 and group 5 ($p<0.05$). However, there was no significant difference for water intake at day 20 (Table 4.1).

**Table 4.1** Food intake (g/day) and water intake (ml/day) in five experimental groups

| | Period | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|---|
| Food intake | Day 1 | 21.1±1.6 | 19.3±1.8 | 19.5±1.5 | 22.6±1.8 | 15.0±0.8* |
| | Day 11 | 25.5±1.3 | 21.3±1.8 | 19.1±1.9 | 24.8±2.1 | 19.6±3.0 |
| | Day 19 | 23.3±0.8 | 21.5±0.8 | 18.3±2.2* | 22.7±0.7 | 20.9±1.5 |
| Water intake | Day 0 | 20.5±2.3 | 33.6±6.7 | 29.1±7.7 | 20.0±2.2 | 17.9±2.5 |
| | Day 10 | 37.9±4.3† | 52.9±4.1* | 23.3±6.6# | 37.8±6.6 | 27.4±3.9# |
| | Day 20 | 28.8±3.8 | 39.5±2.9 | 24.5±5.5 | 35.2±7.0 | 34.8±5.5† |

The data are shown as mean±SEM.

* means significant difference when compared with group 1 ($p<0.05$)

# means significant difference when compared with group 2 ($p<0.05$)

† means significant difference when compared with day 0 ($p<0.05$)

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***Plasma creatinine and BUN***

No significant difference in plasma creatinine concentrations along with experimental period in all groups except group 2 which was elevated on day 21 when compared with baseline ($p<0.05$) (Table 4.2, Figure 4-1). The BUN also temporary increased on day 10 in group 2 and 3.and returned to normal at day 21 when compared with baseline.

**Table 4.2** Plasma creatinine (mg/dl) and blood urea nitrogen (mg/dl) at day 1, 11 and 21.

| | Day | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| PCr | D1 | 0.25±0.02 | 0.30±0.02 | 0.33±0.02 | 0.30±0.02 | 0.30±0.02 |
| | D11 | 0.33±0.04 | 0.45±0.03 | 0.43±0.08 | 0.43±0.10 | 0.31±0.04 |
| | D21 | 0.53±0.15 | 0.59±0.11† | 0.31±0.03 | 0.40±0.04 | 0.37±0.04 |
| BUN | D1 | 21.1±1.4 | 16.9±1.3 | 16.7±0.6 | 16.6±1.1 | 14.7±0.8 |
| | D11 | 20.4±0.67 | 29.0±4.2† | 23.7±2.1† | 22.2±2.8 | 17.0±1.7 |
| | D21 | 21.1±5.5 | 25.7±3.7 | 17.6±1.0 | 20.8±3.5 | 16.0±1.9 |

The data are shown as mean±SEM.

† means significant difference when compared with day 0 ($p$<.05).

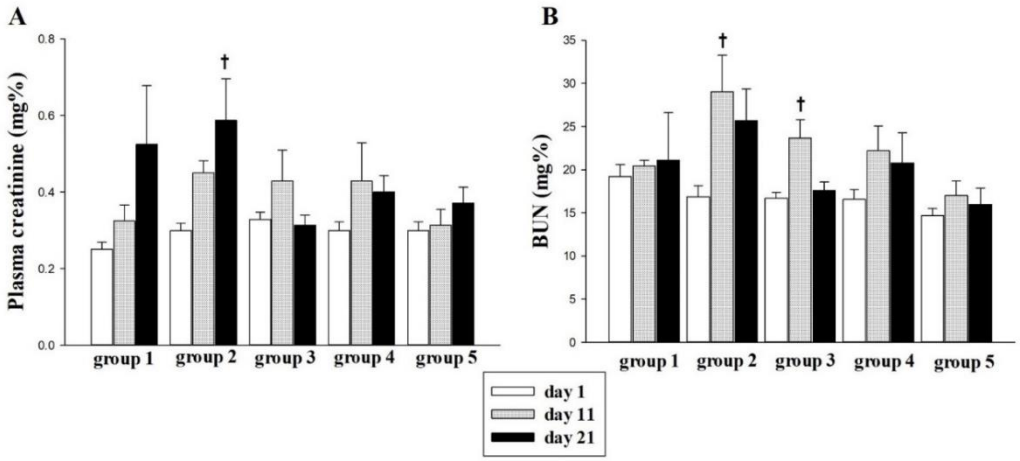


**Figure 4-1** Mean values of plasma creatinine (A) and BUN (B) in five experimental groups. † means significant difference when compared with day 0 ($p$<0.05).

### *Hematocrit and blood pressure*

The hematocrit among groups were not significant difference both on day 0 and day 10. However, the indirect blood pressure, the systolic blood pressure in group

2 at day 10 of the experiment was significantly elevated at day 10 when compared with day 0 on the same group (Table 4.3).

**Table 4.3** The hematocrit (%) and systolic blood pressure (mmHg) in each group on day 0 and day 10.

| | Period | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| Hct | D0 | 45.1±0.5 | 45.6±0.4 | 46.3±0.5 | 45.4±0.4 | 45.6±0.4 |
| | D10 | 45.7±1.0 | 45.1±0.7 | 46.4±0.3 | 45.9±0.6 | 45.7±0.6 |
| SBP | D0 | 138±6 | 124±4 | 126±9 | 120±5 | 132±9 |
| | D10 | 149±6 | 140±8$^{\dagger}$ | 126±7 | 127±2 | 124±7 |

Data are shown as mean±SEM.

$^{\dagger}$ means significant difference in the same group when compared with day 0 ($p$<0.05).

## B. Renal hemodynamic parameters from renal clearance study

Renal hemodynamics data were obtained on day 21 using clearance study. Mean arterial pressure (MAP) and systolic blood pressure (SBP) were no differences among groups. The hematocrit (Hct) after renal clearance study and the average of urine flow rates (V) between renal clearance study were also not different among group (Table 4.4). However, GFR was the lowest in group 2. The reduction of GFR in group 2 was the highest when compared with group 1, 4 and 5 ($p$<0.05) (Figure 4-2). The effective renal plasma flow (ERPF) (Figure 4-2) and effective renal blood flow (ERBF) (Table 4.4) were slightly lower in group 2 when compared with another groups. The renal vascular resistance (RVR) was slightly increased although not significance.

**Table 4. 4** The systolic blood pressure (mmHg), mean arterial blood pressure (mmHg), ERBF (µl/g/min) and urine volume (µl/g/min) in each group on day 21.

| | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|
| SBP | 125±6 | 141±9 | 124±9 | 129±11 | 148±6 |
| MAP | 107±5 | 119±11 | 107±9 | 111±11 | 130±5 |
| ERBF | 38.2±5.2 | 28.6±4.8 | 37.4±9.6 | 39.7±3.7 | 39.6±6.2 |
| V | 0.289±0.057 | 0.178±0.026 | 0.174±0.0.37 | 0.291±0.058 | 0.231±0.024 |

Data are shown as mean±SEM.

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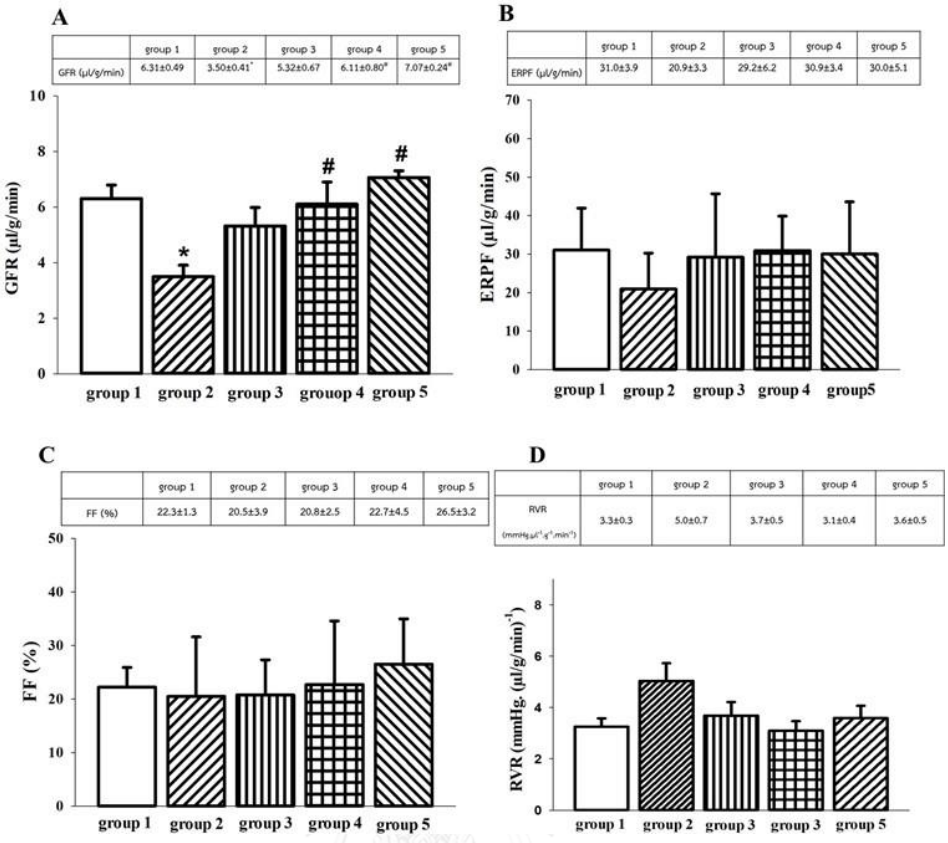


**Figure 4-2** Renal hemodynamic parameters; GFR (A), ERPF (B), FF (C) and RVR (D), * means significant difference when compared with group 1 ($p$<0.05) # means significant difference when compared with group 2 ($p$<0.05)

## C. Tubular reabsorption of water and sodium from lithium clearance study

The reduction in water and sodium reabsorption at proximal tubule by 46% were found in HLP-treated rats when compared with control rats ($p$<0.05) (Table 4.5, Figure 4-3). Supplement with vitamin E, vitamin C or its combination improved water and sodium reabsorption at proximal tubule. However, both water and sodium reabsorption at the distal tubules were not differences among groups.

**Table 4. 5** The water and sodium transportation and reabsorption from lithium clearance study.

| Parameters | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|
| 1. $C_{In}$ - $C_{Li}$ | 5.15±0.23 | 2.80±0.39* | 4.61±0.57 | 5.20±0.82# | 6.10±0.30# |
| 2. ($C_{In}$ - $C_{Li}$) x $P_{Na}$ | 0.75±0.04 | 0.39±0.06* | 0.65±0.08 | 0.73±0.11# | 0.84±0.03# |
| 3. $C_{Li}$ - V | 0.87±0.28 | 0.55±0.16 | 0.54±0.21 | 0.62±0.09 | 0.73±0.10 |
| 4. ($C_{Li}$ – $C_{Na}$) x $P_{Na}$ | 0.11±0.03 | 0.07±0.02 | 0.09±0.04 | 0.10±0.02 | 0.11±0.02 |
| 5. $C_{Li}$ | 402±93 | 292±81 | 264±95 | 369±56 | 354±32 |
| 6. $FE_{Li}$ | 17.3±3.2 | 21.7±3.9 | 12.8±3.1 | 17.1±4.5 | 14.0±1.7 |
| 7. $C_{Na}$ / $C_{li}$) | 0.31±0.05 | 0.28±0.05 | 0.22±0.05 | 0.22±0.03 | 0.17±0.03 |
| 8. (V / $C_{li}$) | 0.32±0.06 | 0.32±0.06 | 0.33±0.06 | 0.33±0.04 | 0.27±0.04 |

Data are shown as mean±SEM, * means significant difference when compared with group 1 ($p$<0.05), # means significant difference when compared with group 2 ($p$<0.05).

$C_{In}$ - $C_{Li}$ = reabsorption of water in proximal tubule (µl/g/min)

($C_{In}$ - $C_{Li}$) x $P_{Na}$ = reabsorption of sodium in proximal tubule (µEq/g/min)

$C_{Li}$ – V = reabsorption of water in distal tubule (µl/g/min)

($C_{Li}$ – $C_{Na}$) x $P_{Na}$ = reabsorption of sodium in distal tubule (µEq/g/min)

$C_{Li}$ = Delivery of sodium out of proximal tubule (µl/g/min)

$FE_{Li}$ = The fractional excretion of sodium from proximal tubule (%)

$C_{Na}$ / $C_{li}$) = Urinary excretion of sodium from proximal tubule (%)

(V / $C_{li}$) = Urinary excretion of water from proximal tubule (%)

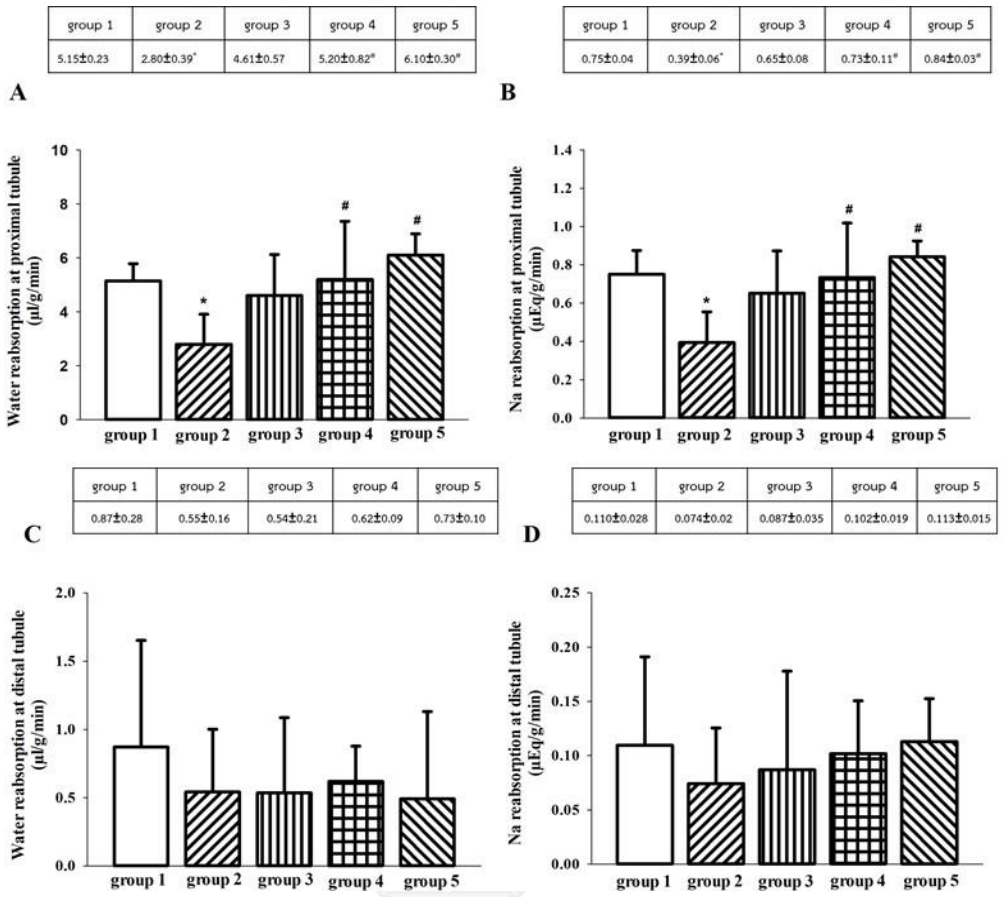


**Figure 4-3** The reabsorption of sodium and water at proximal and distal convoluted tubule (A-D) by using lithium clearance study. * means significant difference as comparable with group 1 ($p<0.05$) # means significant difference as comparable with group 2 ($p<0.05$)

## D. Plasma electrolytes concentrations

Plasma Na, K, Cl, Ca, Mg, and and Ca/Mg were not differences among groups (Table 4.6). Plasma oxalate in HLP-treated rats (group 2-5) had elevated as compare with control group while plasma citrate was changed in a contradictory way. The ratio of plasma oxalate/citrate was the highest in group 2 while supplement with Vit E, Vit

C or their combination tended to be declined. There was no significant difference among the plasma osmolarity from all groups of rats.

**Table 4.6** Plasma concentrations of inorganic substances (mmol/l), zinc (μmol/l), oxalate (μmol/l), citrate (μmol/l) and plasma osmolarity (mOsm/l) obtained on day 21 in 5 groups of rats.

| Parameters | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|
| Na | 154±6 | 146±4 | 143±3 | 149±4 | 141±1 |
| K | 5.5±0.5 | 4.7±0.19 | 5.5±0.5 | 4.6±0.4 | 4.7±0.2 |
| Cl | 101±2 | 103±3 | 102±1 | 102±2 | 105±2 |
| Ca | 3.1±0.3 | 2.3±0.2 | 2.6±0.3 | 2.6±0.2 | 2.6±0.2 |
| Mg | 1.2±0.1 | 0.9±0.1 | 1.3±0.3 | 0.9±0.1 | 0.9±0.1 |
| Ca/Mg | 2.6±0.1 | 2.6±0.1 | 2.3±0.3 | 2.8±0.1 | 2.9±0.2 |
| Zn | 30±3 | 20±5 | 24±4 | 33±3 | 29±2 |
| Oxalate | 48±20 | 152±22* | 168±34* | 212±34* | 121±18 |
| Citrate | 356±148 | 227±137 | 113±24 | 128±19 | 121±27 |
| Ox/Cit | 0.2±0.1 | 2.3±1.1 | 1.3±0.3 | 1.8±0.4 | 0.9±0.2 |
| POsm | 322±3 | 329±3 | 330±11 | 333±5 | 345±6 |

The data are shown as mean±SEM. * means significant difference when compared with group 1 using One-way ANOVA ($p<0.05$).

## E. Renal handling of organic and inorganic substances

The urinary excretion of organic and inorganic substances ($U_xV$) were shown in Table 4.7 and 4.8 while the risk indices were shown in table 4.9. At day 20, the oxalate

excretion was significantly higher in group 2, 4 and 5 as comparable with control group. However, group 3 had lower oxalate excretion compared with group 2 ($p$<0.05). At day 10, urine citrate excretion in group 2 declined significantly compared to day 0 and compared with group 5. The urinary excretions of Na, K, Cl, Ca, Mg and Zn were not different among each group. The Ca/Mg was unchanged in all groups. Moreover, the fractional excretion (FE) of Na, K, Cl, Ca, Mg and Zn (%) on day 20 were not different among each group (Table 4.10).

**Table 4.7** The urinary excretion of oxalate and citrate (μmol/day) in all groups throughout the experimental period.

| Parameters | Period | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| $U_{Ox}V$ | D0 | 13.6±5.5 | 10.7±2.5 | 15.8±3.4 | 13.4±1.4 | 11.8±3.5 |
| | D10 | 7.8±1.6 | 57.9±9.1$^{*,\dagger}$ | 23.2±5.8 | 68.2±26.9$^{*,\dagger}$ | 24.6±3.8 |
| | D20 | 16.5±6.7 | 56.6±9.6$^{*,\dagger}$ | 17.6±7.2$^{\#}$ | 77.0±16.3$^{*,\dagger}$ | 37.8±8.9$^{*,\dagger}$ |
| $U_{Cit}V$ | D0 | 45.2±12.8 | 32.5±6.5 | 37.7±8.9 | 32.8±5.3 | 36.5±12.6 |
| | D10 | 32.1±10.6 | 11.9±3.8$^{\dagger}$ | 20.0±9.3 | 17.0±7.3 | 46.7±11.8$^{\#}$ |
| | D20 | 41.7±7.9 | 20.3±5.0 | 15.8±8.4 | 23.3±4.0 | 31.8±6.7 |

Data are shown as mean±SEM.

$^{*}$ means significant difference when compared with group 1 ($p$<0.05)

$^{\#}$ means significant difference when compared with group 2 ($p$<0.05)

$^{\dagger}$ means significant difference when compared with day 0 ($p$<0.05)

**Table 4.8** The urinary excretion of inorganic substances including Na, K, Cl, Ca, Mg and Zn in all groups throughout the experimental period.

| | Period | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| $U_{Na}V$ | D0 | 2.8±0.7 | 1.9±0.2 | 1.6±0.4 | 1.8±0.3 | 1.6±0.3 |
| (mmol/day) | D10 | 2.2±0.6 | 2.4±0.2 | 1.3±0.3 | 2.3±0.7 | 2.2±0.2 |
| | D20 | 2.1±0.3 | 2.2±0.3 | 1.6±0.5 | 2.5±0.3 | 2.6±0.5 |
| $U_{K}V$ | D0 | 1.3±0.3 | 0.9±0.1 | 0.7±0.2 | 1.3±0.2 | 1.1±0.3 |
| (mmol/day) | D10 | 1.1±0.2 | 1.3±0.2 | 0.7±0.1 | 1.2±0.3 | 1.2±0.1 |
| | D20 | 1.0±0.1 | 1.0±0.2 | 0.8±0.2 | 1.3±0.2 | 1.5±0.3 |
| $U_{Cl}V$ | D0 | 0.4±0.1 | 0.5±0.1 | 0.2±0.1 | 0.3±0.1 | 0.2±0.0 |
| (mmol/day) | D10 | 0.5±0.1 | 1.5±0.4 | 0.2±0.1 | 0.2±0.1 | 0.2±0.1 |
| | D20 | 0.3±0.1 | 0.8±0.3 | 0.2±0.1 | 0.2±0.1 | 0.3±0.1 |
| $U_{Ca}V$ | D0 | 7.6±1.6 | 9.4±2.4 | 5.1±2.6 | 3.9±1.1 | 4.1±1.5 |
| (µmol/day) | D10 | 15.6±3.9 | 3.3±0.8 | 4.0±1.8 | 8.7±6.6 | 5.8±2.1 |
| | D20 | 15.3±5.2 | 7.4±3.9 | 3.5±1.7 | 4.6±2.1 | 4.1±2.5 |
| $U_{Mg}V$ | D0 | 42.3±12.4 | 64.5±32.9 | 27.8±12.3 | 43.7±21.7 | 51.9±26.1 |
| (µmol/day) | D10 | 70.0±20.9 | 44.8±11.8 | 24.8±7.3 | 49.4±22.6 | 24.6±11.1 |
| | D20 | 48.9±13.3 | 43.6±16.5 | 14.9±6.4 | 28.1±6.1 | 7.8±6.5 |
| $U_{Zn}V$ | D0 | 0.7±0.2 | 0.6±0.2 | 0.4±0.1 | 0.7±0.4 | 0.6±0.2 |
| (µmol/day) | D10 | 1.2±0.4 | 26.4±9.8 | 3.3±2.0 | 4.1±2.5 | 7.6±3.6 |
| | D20 | 14.6±13.7 | 16.1±8.4 | 10.1±6.6 | 12.0±6.8 | 26.6±19.5 |

Data are shown as mean±SEM.

* means significant difference when compared with group 1 ($p<0.05$)

# means significant difference when compared with group 2 ($p<0.05$)

† means significant difference when compared with day 0 ($p<0.05$)

**Table 4.9** The risk indices for prediction of urinary supersaturation including the ratio of Ca/Mg, Ox/Cit and Tiselius index.

| | Period | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| Ca/Mg | D0 | 1.0±0.6 | 0.6±0.2 | 0.7±0.4 | 0.4±0.1 | 0.5±0.2 |
| | D10 | 2.1±1.7 | 0.1±0.0 | 0.7±0.6 | 0.3±0.2 | 1.1±0.5 |
| | D20 | 1.3±0.9 | 0.5±0.3 | 0.7±0.4 | 0.2±0.1 | 2.1±1.0 |
| Ox/Cit | D0 | 0.3±0.1 | 0.3±0.1 | 0.5±0.2 | 0.5±0.2 | 0.3±0.1 |
| | D10 | 0.3±0.1 | 13.8±5.0 | 3.7±1.3 | 17.8±11.3 | 0.9±0.4 |
| | D20 | 0.4±0.1 | 4.3±1.6 | 2.2±0.6 | 4.0±1.1 | 1.4±0.3 |
| Tiselius index | D0 | 3.8±1.7 | 2.7±0.7 | 3.4±0.8 | 3.3±0.7 | 3.3±1.9 |
| | D10 | 2.3±0.9 | 3.2±0.9 | 12.5±8.3 | 5.7±3.2 | 7.1±2.4 |
| | D20 | 4.3±2.1 | 2.8±1.0 | 4.1±1.6 | 2.2±0.4 | 2.8±6.5 |

Data are shown as mean±SEM.

**Table 4.10** Fractional excretion (FE) of Na, K, Cl, Ca, Mg and Zn (%) on day 20

| | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|
| FE Na | 1.4±0.5 | 1.1±0.4 | 0.6±0.1 | 0.7±0.2 | 0.8±0.1 |
| FE K | 20.3±7.0 | 16.3±4.9 | 7.5±1.0 | 11.8±3.2 | 13.7±1.9 |
| FE Cl | 12.6±6.5 | 6.2±1.6 | 6.6±4.2 | 13.3±8.5 | 6.4±2.0 |
| FE Ca | 0.5±0.2 | 0.2±0.1 | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 |
| FE Mg | 2.8±1.0 | 3.5±1.0 | 1.7±1.3 | 1.3±0.3 | 0.5±0.5 |
| FE Zn | 18.7±15.2 | 31.3±6.4 | 63.7±58.9 | 13.5±7.7 | 55.1±35.5 |

Data are shown as mean±SEM.

## *Renal handling of calcium and magnesium*

The filter load and tubular reabsorption of calcium and magnesium were drastically lower in group 2 as comparable with group 1. However, rats in group 4 and 5 mostly recovered the capacity of tubular reabsorption (Figure4-4).

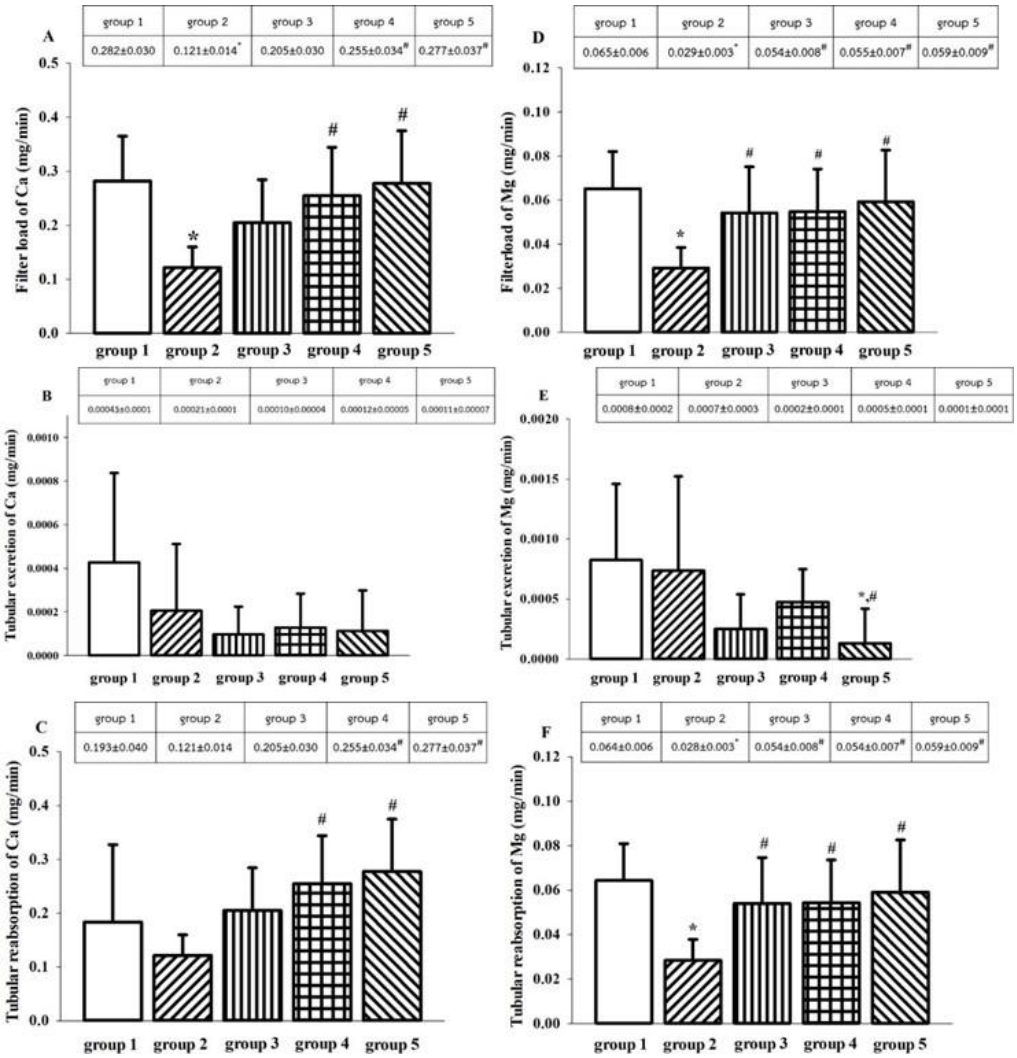


**Figure 4-4** Renal handling of calcium and magnesium. Filter load of Ca and Mg (A, D), tubular excretion of Ca (B, E) and tubular reabsorption of Ca (C, F). *means significant difference when compared with group 1 ($p<0.05$), # means significant difference when compared with group 2 ($p<0.05$).

## *Renal handling of oxalate and citrate*

Interestingly, the filter load, tubular reabsorption and tubular excretion were drastically elevated in group 4 while the renal ability of filter load, tubular reabsorption and tubular excretion of citrate in HLP-treated groups (group 2-5) were diminished as shown in Figure 4-5.

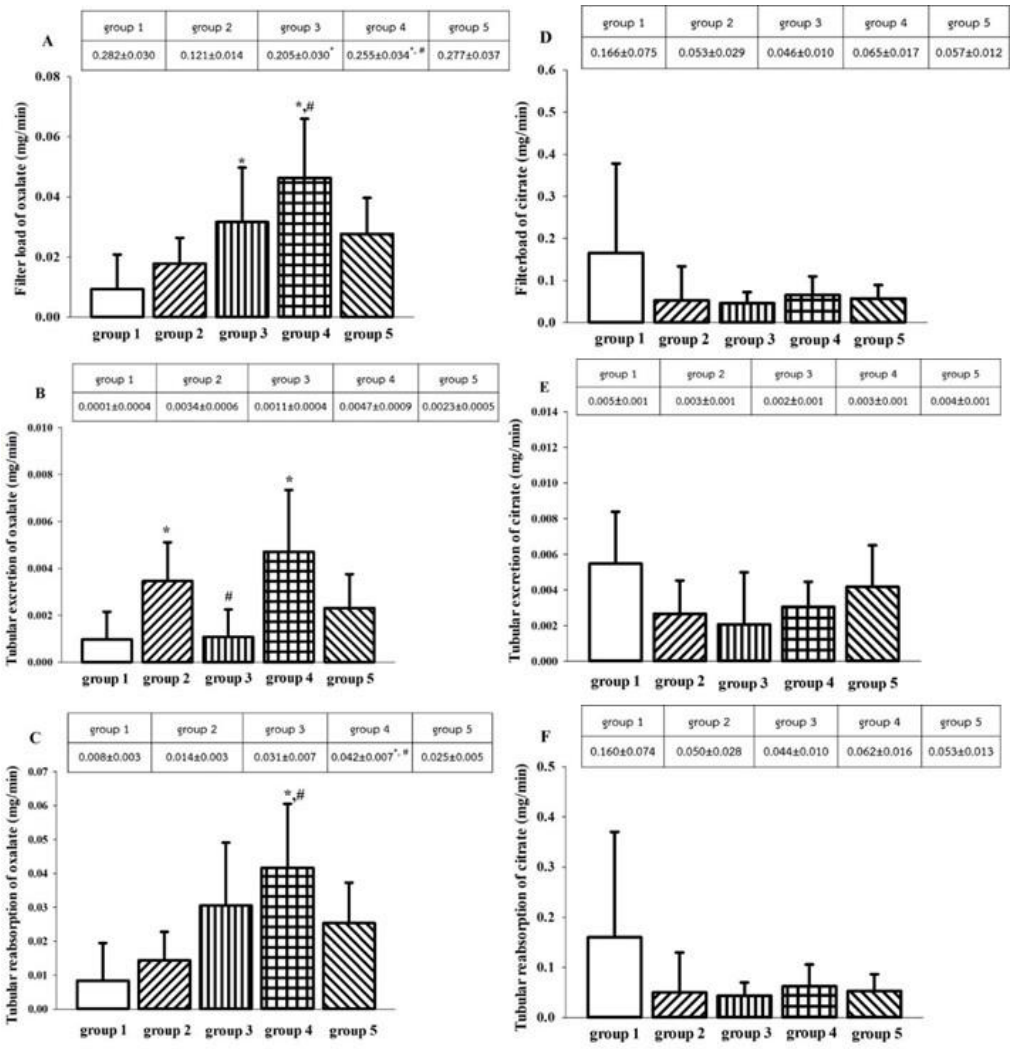


**Figure 4-5** Renal handling of oxalate and citrate in five groups of experiments. * means significant difference when compared with group 1 ($p<0.05$), # means significant difference when compared with group 2 ($p<0.05$).

## *Renal handling of zinc*

The ability of renal handling of trace element, zinc, was reduced in group 2 but not significant difference when compared with control group. However, vitamin C treated group had the highest filter load and tubular reabsorption of zinc (Figure 4-6).

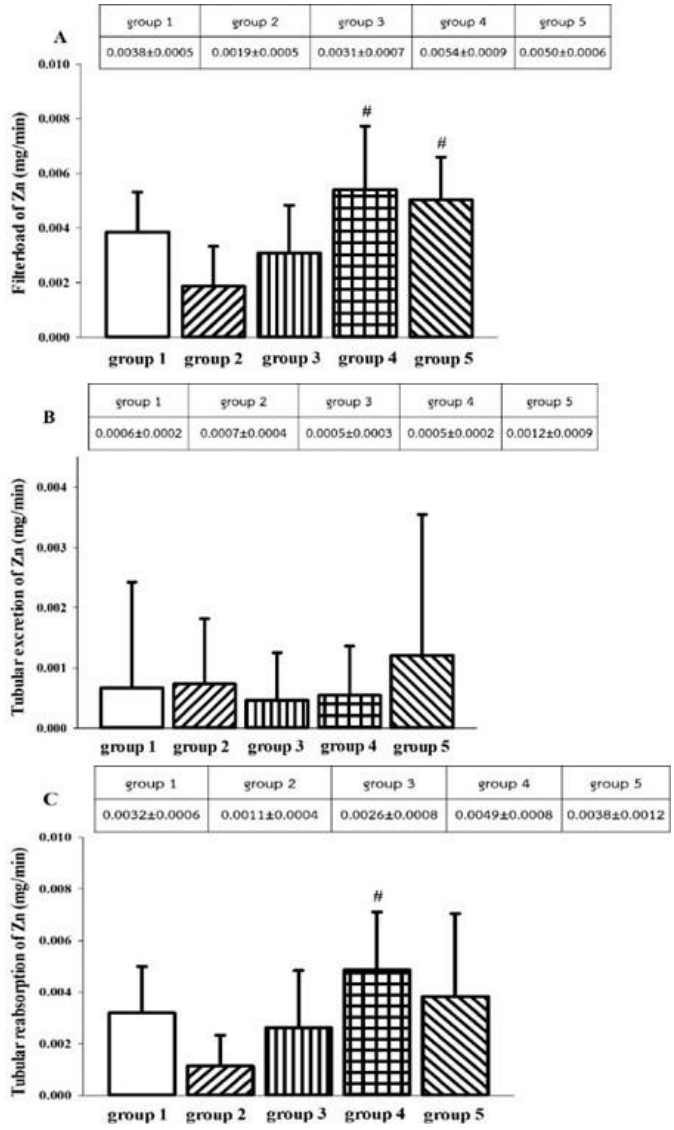


**Figure 4-6** The filter load, tubular reabsorption and tubular excretion of zinc in all groups of the study. # means significant difference when compared with group 2 ($p<0.05$).

## F. Urinalysis results

The results of urine protein excretion and urine pH were shown in Table 4.11. Protein excretion was elevated significantly on day 10 in group 2 as comparable with control group while supplement with vitamin E in group 3 reduced urinary protein excretion when compared with group 2. The urine pH and urine volume were not significant alteration among study groups throughout the period of the study. Moreover, the urine specific gravity (USG) in group 1-5 was unchanged among experimental groups (USG of group 1-5 on day 20; $1.023 \pm 0.003$, $1.019 \pm 0.001$, $1.030 \pm 0.005$, $1.024 \pm 0.002$ and $1.022 \pm 0.002$). All HLP-treated groups had significant higher crystal deposition than control on day 10 and day 20 (Figure 4-7). The highest crystal score was observed in group 2, while vitamin E supplement appeared to alleviate crystal score in urine sediment (group 3 and 5) (Figure 4-8 – 4-13).

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**Table 4.11** Urinary protein excretion (mg/day), urine pH and 24 hr urine volume (ml/day) from urinalysis

| | Period | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| Protein excretion | D0 | 69.1±16.2 | 81.5±16.3 | 78.7±31.9 | 64.4±18.9 | 64.3±12.1 |
| | D10 | 61.9±11.5 | 112.2±13.3* | 53.6±12.0# | 68.6±19.5 | 67.8±14.4 |
| | D20 | 62.4±9.9 | 61.5±10.4 | 36.2±9.9 | 60.8±9.9 | 65.6±9.4 |
| Urine pH | D0 | 6.75±0.25 | 7.25±0.25 | 7.86±0.40 | 7.43±0.37 | 7.29±0.42 |
| | D10 | 6.75±0.37 | 6.75±0.37 | 6.86±0.40 | 6.71±0.42 | 7.43±0.37 |
| | D20 | 7.13±0.35 | 6.63±0.26 | 7.29±0.36 | 6.71±0.29 | 7.29±0.18 |
| 24 hr urine volume | D0 | 16.5±2.2 | 18.2±2.6 | 11.5±3.5 | 11.5±1.9 | 11.5±2.3 |
| | D10 | 23.4±4.1 | 28.5±4.2 | 9.7±2.9 | 17.7±6.2 | 12.3±2.2 |
| | D20 | 15.0±2.9 | 20.2±3.9 | 11.0±3.3 | 18.3±3.9 | 17.9±3.3 |

Data are shown as mean±SEM

* means significant difference when compared with group 1 ($p$<0.05), # means significant difference when compared with group 2 ($p$<0.05).

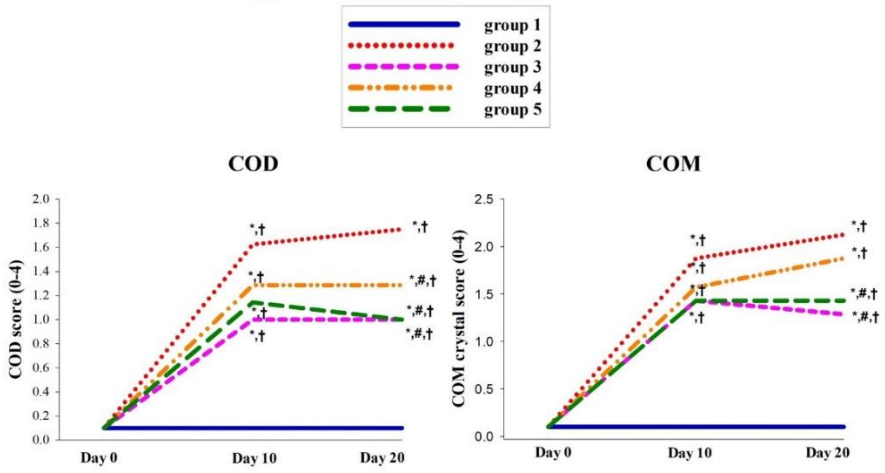


**Figure 4-7** COD and COM crystal score from urinalysis study of rats in group 1 – 5. $^{*}p < 0.05$ when compared with group 1, $^{\#}p < 0.05$ when compared with group2, $^{\dagger}p < 0.05$ in the same group when compared with day 0.

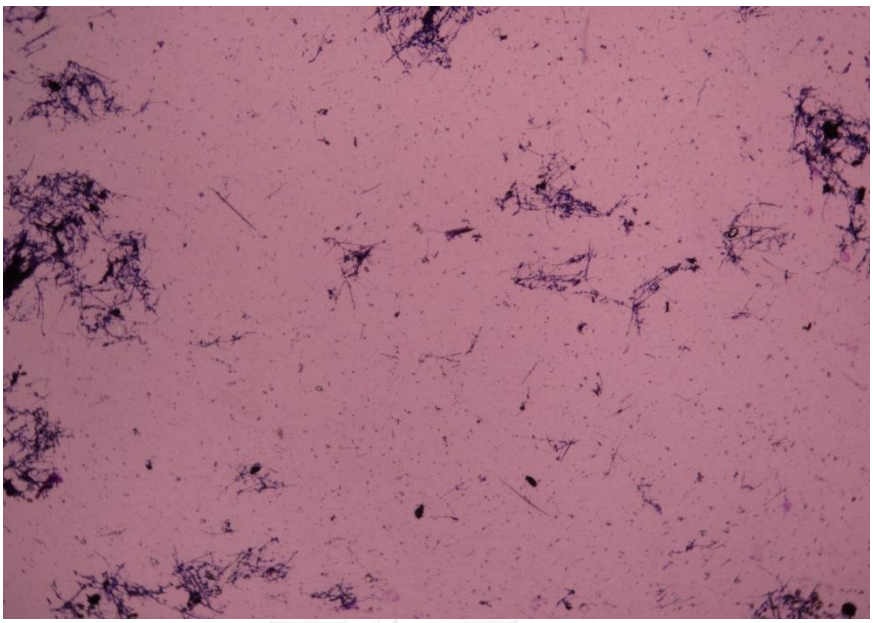

**Figure 4-8** The urine sediment from control group on day 10 (magnification x10). There was no remarkable crystals, casts and cells.

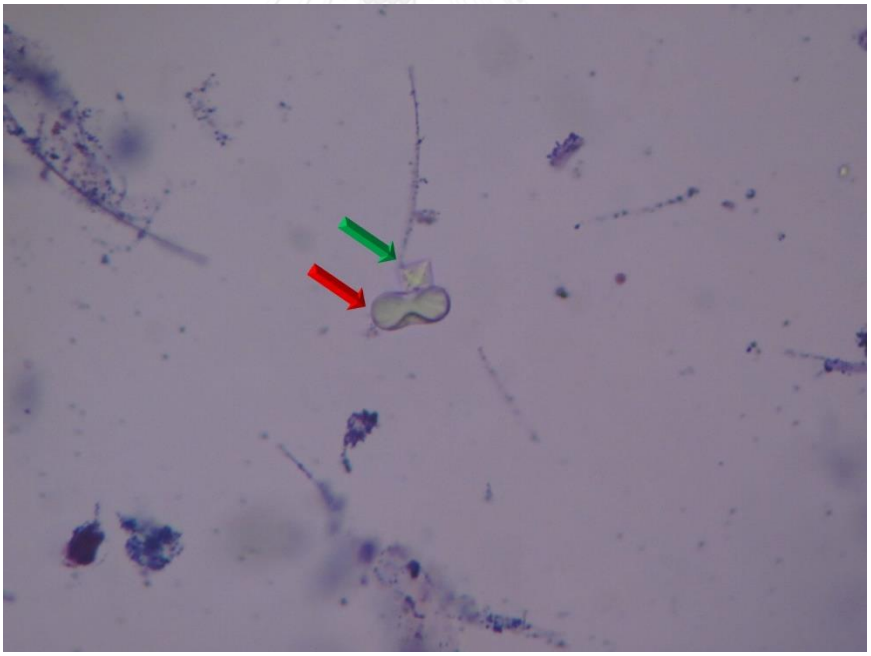

**Figure 4-9** The calcium oxalate monohydrate (COM, red arrow) and calcium oxalate dihydrate (COD, green arrow) revealed from the urine sediment on day 10 of HLP-treated rat (group 2) (x40).

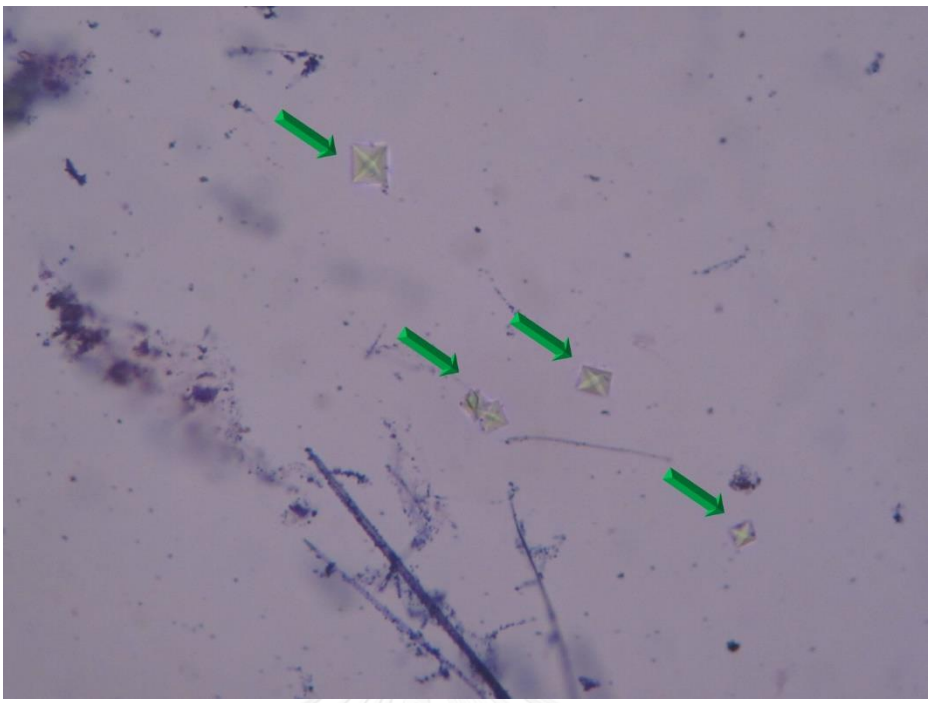

**Figure 4-10** The COD crystal in urine sediment from group 2 on day 20 (green arrow, x40).

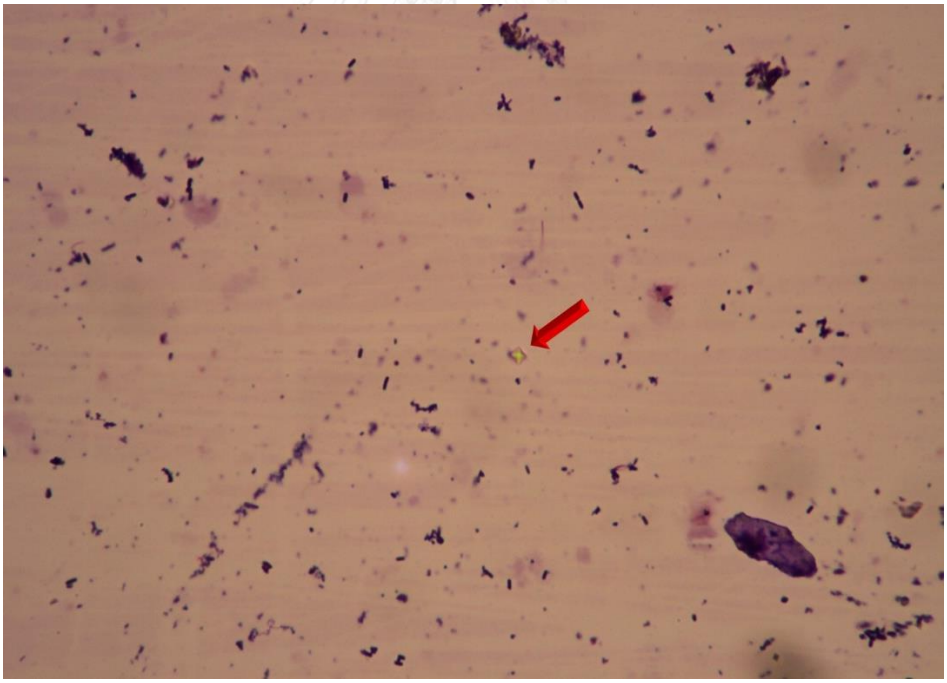

**Figure 4-11** The COD crystal in urine sediment from group 3 on day 20 (red arrow, x40).

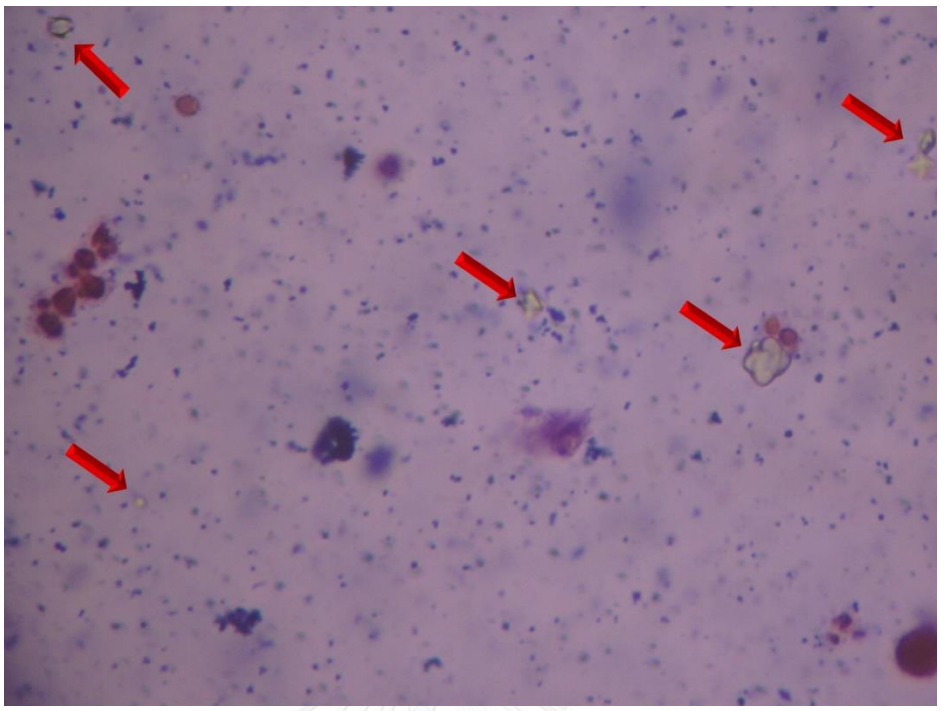

**Figure 4-12** The COD and COM crystal from the urine sediment of group 4 on day 20 (x40).

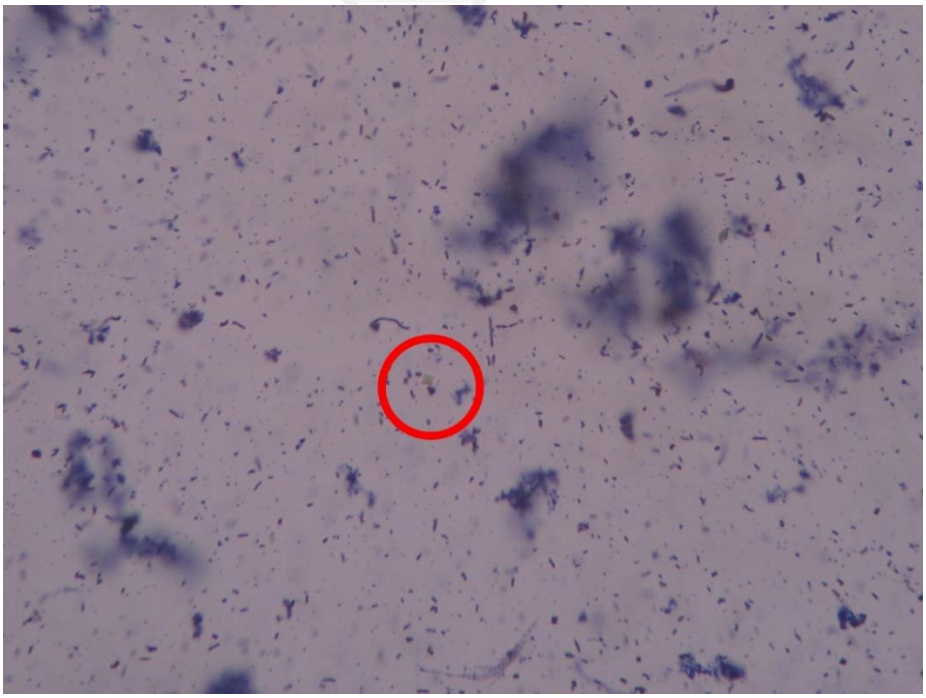

**Figure 4-13** A tiny COD (in the middle of red circle) from urine sediment in group 5 on day 20.

## G. Oxidative stress parameters in urine and plasma

The levels of lipid peroxidation product, MDA, in plasma and urine sample were shown in table 12. At the end of the experiment, group 2 exhibited significantly higher PMDA concentration than control (Table 4.12), with no difference in excretion of urine MDA detected among groups. On the contrary, UTAS was the lowest in group 2, while either vitamin C or vitamin E treated-rats appeared to improve UTAS in hyperoxaluric rats (group 3, 4 and 5).

**Table 4.12** Effects of Vit E and Vit C and its combination on PMDA (nmol/ml), UMDA/UCr (nmol/ mg Cr) and UTAS (%)

| | Period | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|---|
| PMDA | D20 | 1.1±0.3 | 3.3±0.5* | 2.2±0.5 | 1.7±0.4 | 2.0±0.3 |
| UMDA/UCr | D0 | 26.2±5.2 | 30.4±7.5 | 25.0±3.5 | 22.6±4.0 | 33.7±4.6 |
| | D10 | 16.0±3.5 | 20.2±4.6 | 22.4±2.8 | 18.4±1.6 | 24.5±2.6 |
| | D20 | 19.0±1.9 | 27.3±9.9 | 24.0±4.5 | 16.9±1.6 | 30.2±5.8 |
| UTAS | D0 | 81.9±1.6 | 80.8±1.2 | 78.4±2.7 | 79.1±1.1 | 80.1±1.0 |
| | D10 | 82.1±1.4 | 23.7±4.8$^{*,\dagger}$ | 81.1±2.7$^{\#}$ | 76.7±3.9$^{\#}$ | 80.2±1.2$^{\#}$ |
| | D20 | 83.0±1.8 | 29.7±4.7$^{*,\dagger}$ | 80.1±1.6$^{\#}$ | 77.7±2.1$^{\#}$ | 78.7±1.0$^{\#}$ |

Data was shown as mean±SEM

$^{*}$ means significant differece when compared with group 1 ($p$<0.05)

$^{\#}$ means significant difference when compared with group 2 ($p$<0.05)

† means significant difference when compared with day 0 ($p$<0.05)

## H. Histopathology results

There was mild remarkable lesion of glomerulus among groups when compared with group 1(Figure 4-14 to 4-18). Importantly, rats in group 2 developed moderate to severe flattened tubular epithelial cells in proximal, distal and collecting duct regions. Antioxidant treatment appeared to alleviate glomerular and tubular injury, as milder Bowman's capsule space and tubular epithelial lesions were observed.



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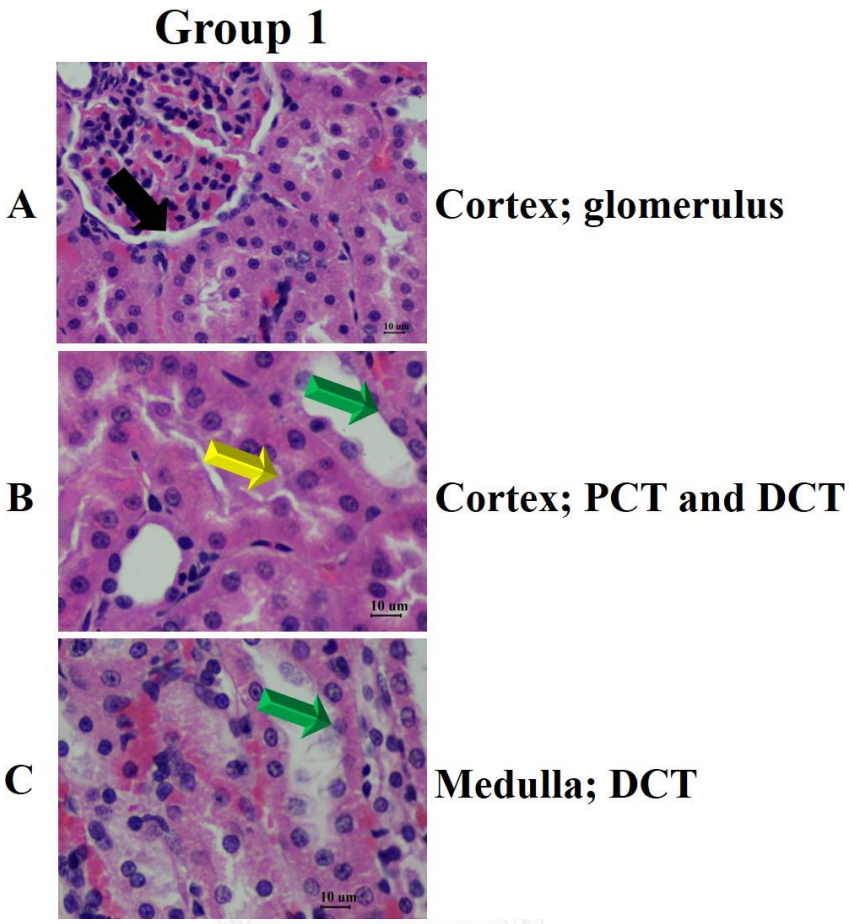


**Figure 4-14** Histopathology pictures of kidney tissue in group 1, no remarkable lesions of both glomerulus and tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space (A); a yellow and green arrow presented proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) in the cortex (B) and medulla (C), respectively (x40, scale bar 10µm).

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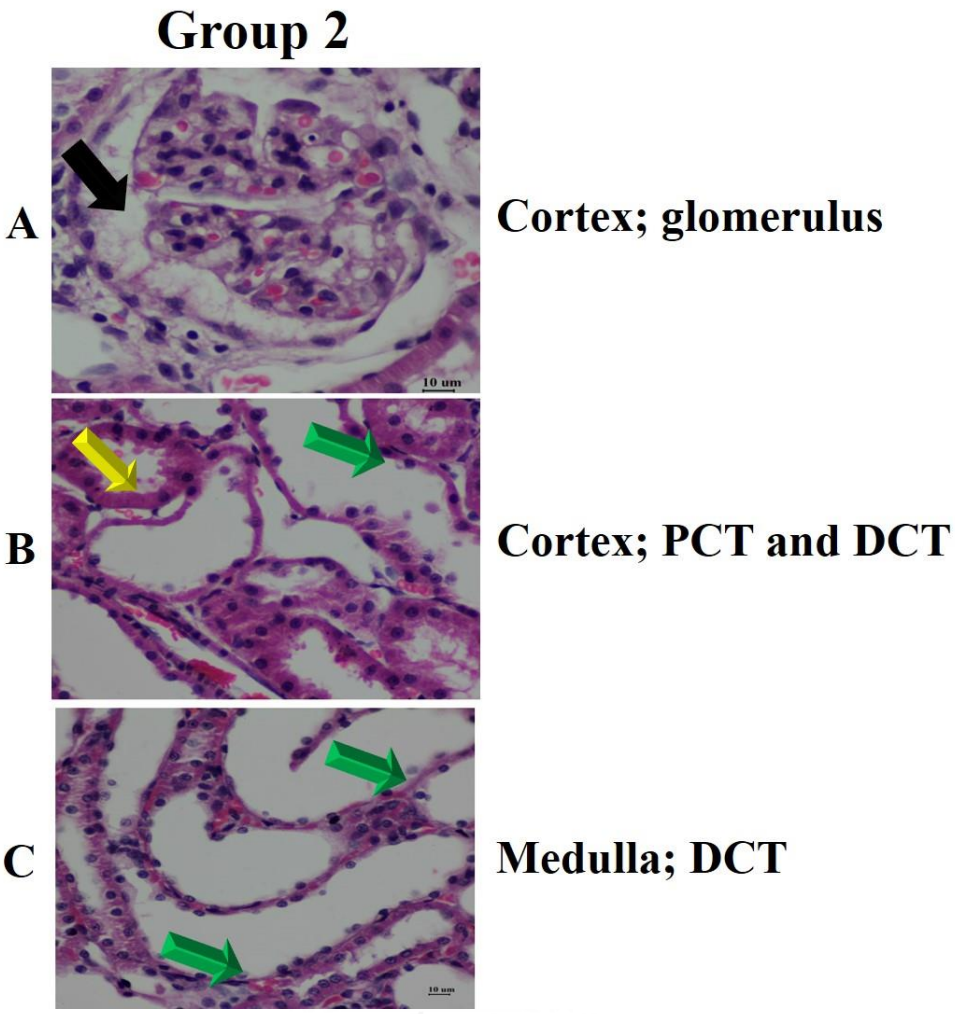


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**Figure 4-15** Histopathology pictures of kidney tissue in group 2, mild lesions of glomerulus and moderate to severe lesions of tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space dilation (A); a yellow and green arrow presented PCT and DCT in the cortex (B) and medulla (C), respectively with marked flatted tubular epithelium and dilated lumen.

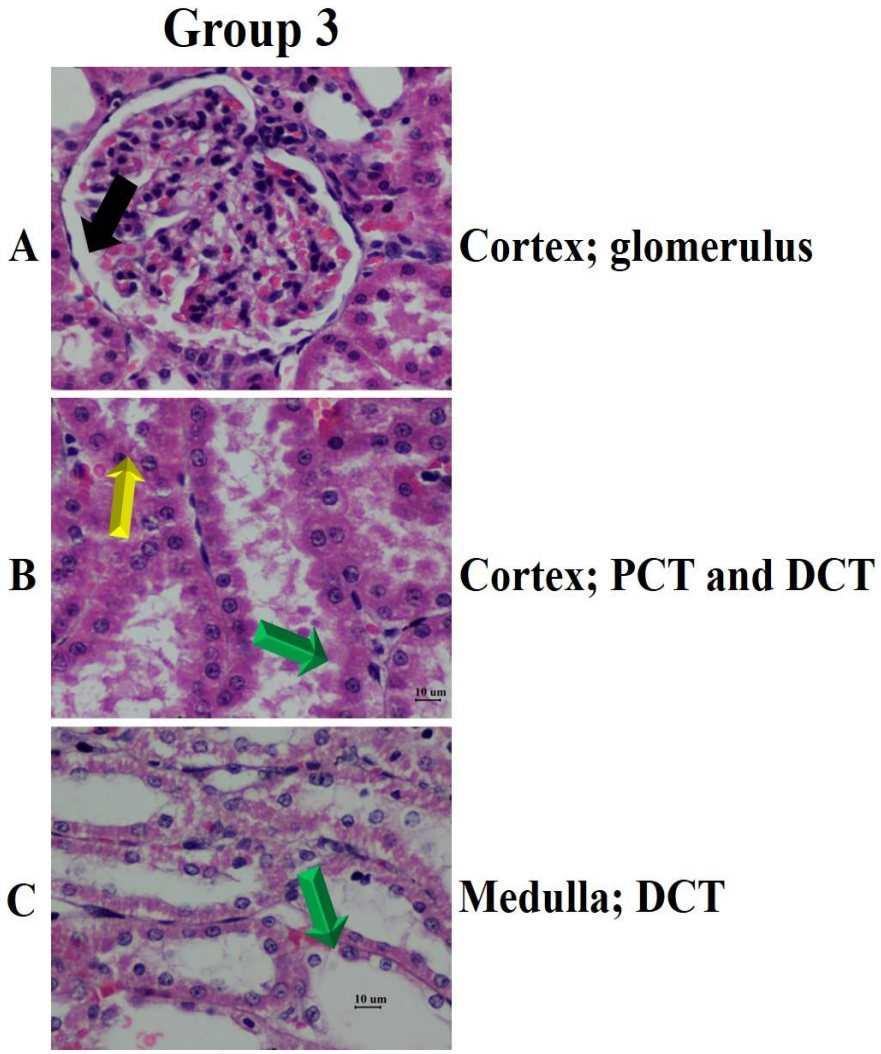


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**Figure 4-16** Histopathology pictures of kidney tissue in group 3, no remarkable lesions of glomerulus with mild lesions of tubules were shown; glomerulus in the cortex and black arrow presented Bowman's capsule space (A); a yellow and green arrow presented proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) in the cortex (B) and medulla (C), respectively (x40, scale bar 10µm). Mild tubular degeneration and dilation were found as comparable with group 2.

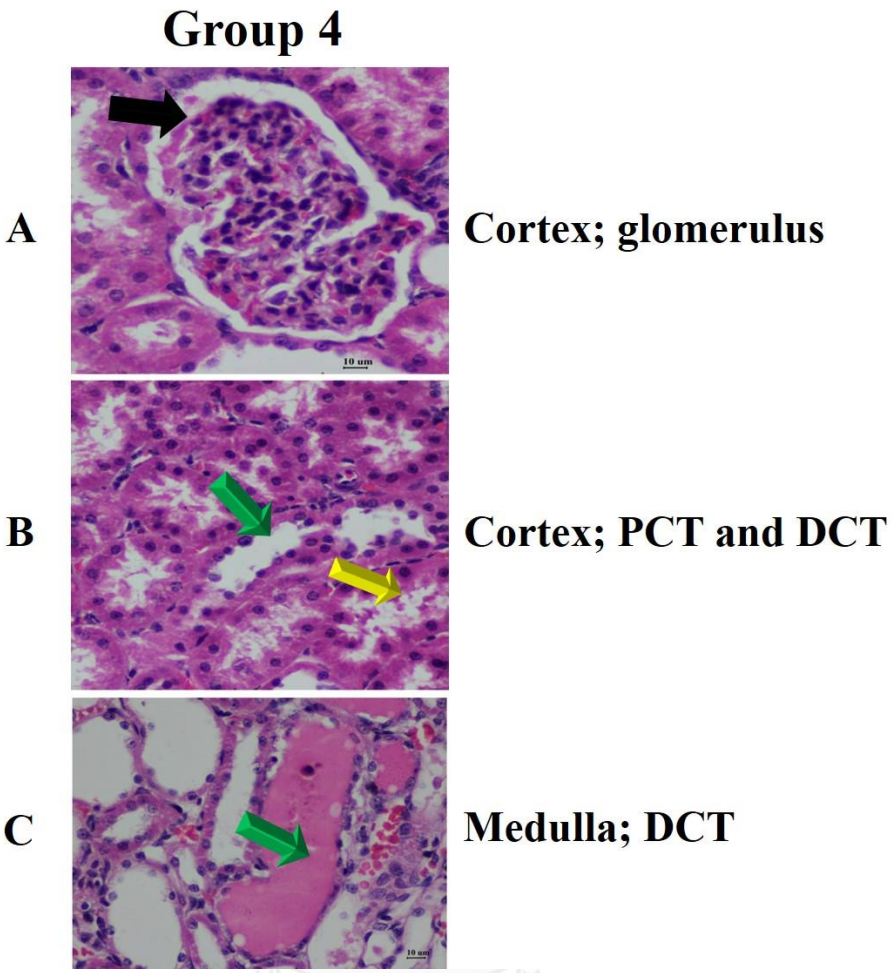


**Figure 4-17** Histopathology pictures of kidney tissue in group 4, no remarkable lesions of glomerulus with mild lesions of tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space (A); a yellow and green arrow presented proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) in the cortex (B) and medulla (C), respectively (x40, scale bar 10μm). Mild lesions of tubular cells and protein casts deposit in lumen were found.

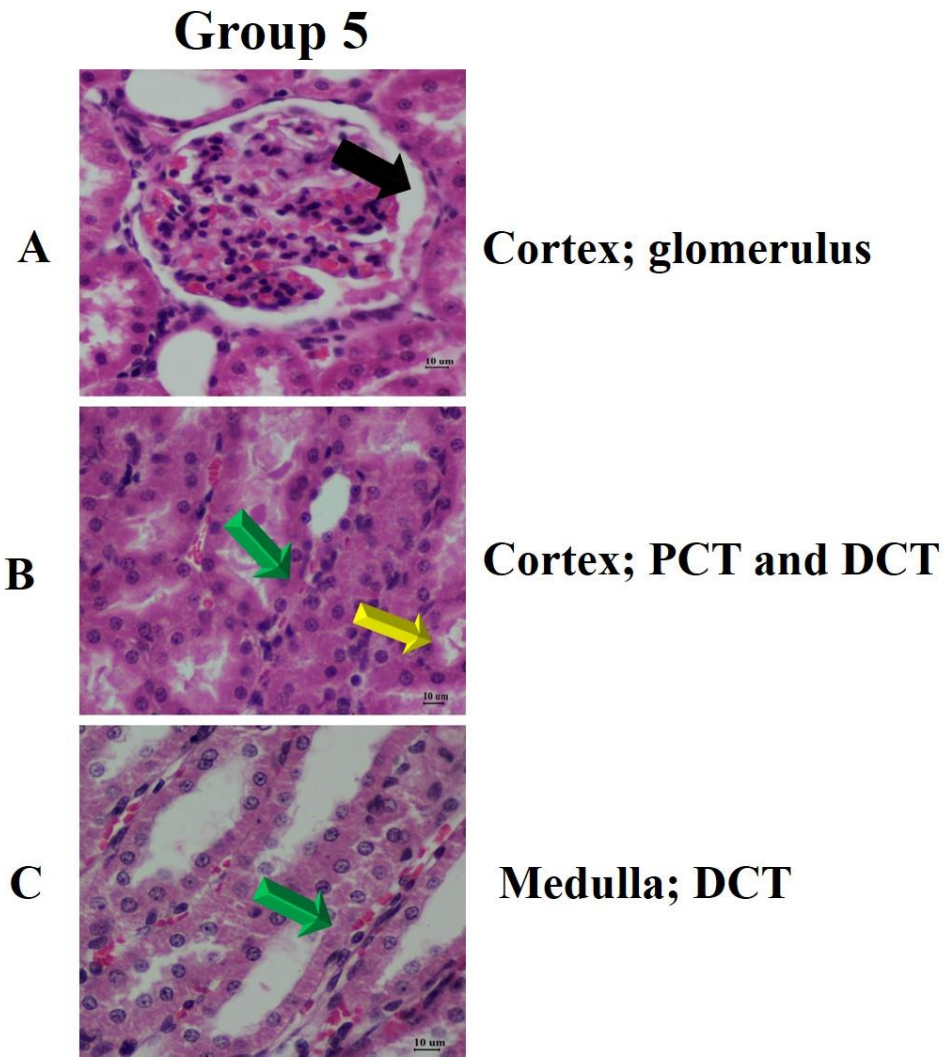


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**Figure 4-18** Histopathology pictures of kidney tissue in group 5, no remarkable lesions of both glomerulus and tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space (A); a yellow and green arrow presented proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) in the cortex (B) and medulla (C), respectively (x40, scale bar 10µm).

## I. The correlation between renal function parameters and oxidative stress

The correlation between renal function parameters, oxalate and oxidative stress were shown in Table 4.13. The GFR, the reabsorption of water and sodium in proximal tubule had negatively correlation with PMDA while the positive correlations were found with UTAS. However, urine MDA/UCr showed no correlation with renal function parameters. The correlation and linear regression were shown in Figure 4-19 to 4-21.

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**Table 4.13** The correlation between parameters (n=37)

| | parameters | Correlation coefficient | *p*-value | n |
|---|---|---|---|---|
| Uoxalate | GFR | r=0.104 | *p*=0.540 | 37 |
| | $C_{In}$ - $C_{Li}$ | r=0.073 | *p*=0.667 | 37 |
| | ($C_{In}$ - $C_{Li}$) x $P_{Na}$ | r=0.062 | *p*=0.714 | 37 |
| Uoxalate | PMDA | r=0.335 | *p*<0.05 | 37 |
| | UMDA | r= 0.144 | *p*= 0.394 | 37 |
| | UTAS | r= -0.326 | *p*<0.05 | 37 |
| PMDA | GFR | r=-0.411 | *p*<0.05 | 37 |
| | $C_{In}$ - $C_{Li}$ | r=-0.369 | *p*<0.05 | 37 |
| | ($C_{In}$ - $C_{Li}$) x $P_{Na}$ | r= -0.363 | *p*<0.05 | 37 |
| UMDA | GFR | r=0.053 | *p*=0.753 | 37 |
| | $C_{In}$ - $C_{Li}$ | r=0.088 | *p*=0.605 | 37 |
| | ($C_{In}$ - $C_{Li}$) x $P_{Na}$ | r=0.039 | *p*=0.821 | 37 |
| UTAS | GFR | r=0.522 | *p*<0.001 | 37 |
| | $C_{In}$ - $C_{Li}$ | r=0.533 | *p*<0.001 | 37 |
| | ($C_{In}$ - $C_{Li}$) x $P_{Na}$ | r=0.549 | *p*<0.001 | 37 |

GFR=glomerular filtration rate; PMDA=plasma malondialdehyde levels; UMDA=urinary MDA/UCr ration; UTAS=urinary total antioxidant status; $C_{In}$ - $C_{Li}$=tubular reabsorption of water at proximal tubule and ($C_{In}$ - $C_{Li}$) x $P_{Na}$= tubular reabsorption of sodium at proximal tubule.

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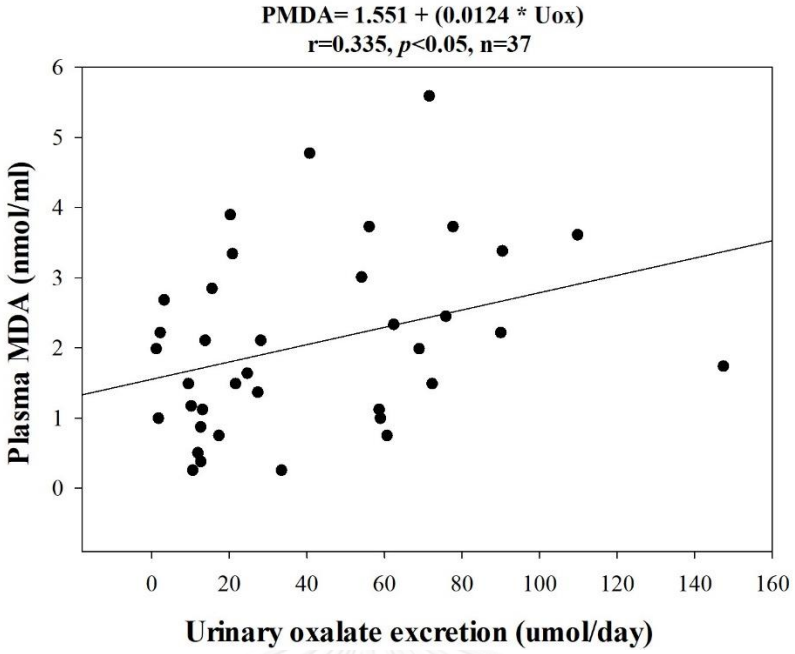


**Figure 4-19** The positive correlation between urinary oxalate excretion and PMDA.

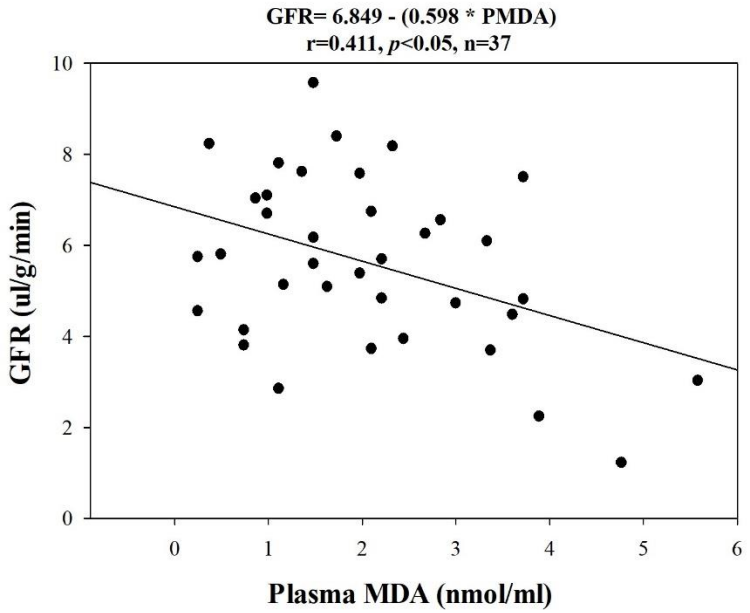


**Figure 4-20** The negative correlation between PMDA and GFR.

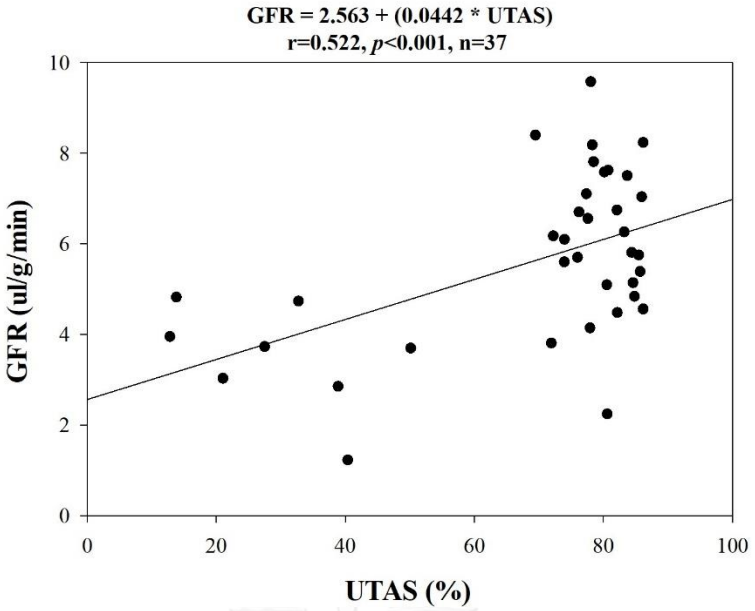


**Figure 4-21** The positive correlation between UTAS and GFR.

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## 2. Study part II

Effects of vitamin E and vitamin C supplement on oxidative stress, Klotho protein levels and Klotho protein mRNA expression in HLP-induced hyperoxaluric rats.

### A. General parameters (BW, food intake, water intake and blood chemistry)

#### *Body weight*

The body weight was not significant difference among experimental group on day 0. At the end of the study, body weight in group 5 had significantly lower ($p$<0.05) when compared with group 1 (Table 4.14).

**Table 4.14** Body weight (grams) in five experimental groups

| | Period | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|---|
| BW | Day 0 | 334±20 | 325±15 | 327±12 | 321±13 | 323±13 |
| | Day 21 | 431±11 | 407±11 | 389±18 | 388±14 | 373±5* |

The data are shown as mean±SEM.

* means significant difference when compared with group 1 ($p$<0.05)

#### *Food and water intake*

Before experiment, there was no significant difference of food and water intake in all study groups at day 0 while group 4 and 5 had lower food intake when compared with group 1 in day 10 of the study. However, at the end of experiment, there was no

significantly difference of food intake among groups. The water intake was significant increase in HLP-treated groups (group 2-5) at day 10 of experiment when compared with baseline in each group ($p$<0.05). However, rats in group 2 had the highest water intake on day 20 as comparable with other groups (Table 4.15).

**Table 4.15** Food intake (g/day) and water intake (ml/day) in five experimental groups

| | Period | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|---|
| Food intake | Day 1 | 24.4±1.6 | 20.2±1.2 | 19.3±2.8 | 21.3±1.5 | 18.7±1.4 |
| | Day 11 | 29.5±0.8 | 25.9±1.8 | 28.6±1.0$^{\dagger}$ | 22.1±1.7* | 16.8±1.6* |
| | Day 19 | 25.1±1.2 | 22.5±0.9 | 22.8±2.2 | 21.9±0.7 | 20.5±0.7 |
| Water intake | Day 0 | 22.5±3.0 | 17.3±1.5 | 15.6±2.6 | 25.0±2.5 | 21.3±2.6 |
| | Day 10 | 25.6±3.2 | 45.2±6.6$^{\dagger}$ | 31.2±3.4$^{\dagger}$ | 41.0±5.9$^{\dagger}$ | 43.4±7.8$^{\dagger}$ |
| | Day 20 | 22.0±6.1 | 51.9±4.8$^{\dagger,*}$ | 41.0±6.2$^{\dagger}$ | 43.8±2.3$^{\dagger}$ | 31.2±1.9 |

The data are shown as mean±SEM.

* means significant difference when compared with group 1 ($p$<0.05)

† means significant difference when compared with day 0 ($p$<.05)

## *Plasma electrolytes concentration and blood pressure*

At the end of the study, the levels of Na, K, Cl, Zn and citrate in plasma and hematocrit in all groups of experimental rats were unaltered while the levels of plasma Ca and Mg trended to be declined (Table 4.16). The systolic blood pressure and plasma oxalate concentrations were increased significantly in HLP-treated rats. However, plasma osmolarity was unchanged among groups.

**Table 4.16** Effects of Vit E, Vit C and its combination on SBP (mmHg), MAP (mmHg), Hct (%), plasma osmolarity (Osm; mOsm/l), plasma inorganic and organic substances (mmol/l), plasma Zn, oxalate and citrate (μmol/l).

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| SBP | 108±8 | 153±12* | 148±13* | 149±8* | 151±11* |
| MAP | 100±5 | 134±9* | 131±12* | 126±8* | 132±9* |
| Hct | 47.3±0.5 | 46.6±0.5 | 46.0±0.5 | 46.8±0.4 | 47.1±0.8 |
| Osm | 290±4 | 289±3 | 294±2 | 294±3 | 285±5 |
| Na | 145±1 | 142±1 | 143±1 | 139±2 | 142±1 |
| K | 6.2±0.4 | 5.5±0.2 | 5.8±0.4 | 4.9±0.2 | 5.4±0.2 |
| Cl | 99±2 | 94±2 | 94±3 | 111±9 | 108±14 |
| Ca | 2.7±0.2 | 2.0±0.3 | 1.8±0.1* | 2.1±0.2 | 2.4±0.3 |
| Mg | 1.2±0.9 | 0.8±0.2 | 0.8±0.1 | 0.8±0.1 | 0.9±0.2 |
| Zn | 26±3 | 17±3 | 14±1 | 24±1 | 18±4 |
| Oxalate | 19±3 | 204±31* | 112±16 | 173±14* | 188±26* |
| Citrate | 120±18 | 144±23 | 156±24 | 75±12 | 156±33 |

The data are shown as mean±SEM.

* means significant difference when compared with group 1 ($p<0.05$)

## B. Renal function parameters

Renal functions were assessed by determined the levels of PCr, BUN and renal handling of organic and inorganic substances as follow:

### *Plasma creatinine and BUN*

In group 2, 4 and 5 the PCr concentration on day 21 was significantly elevated when compared with day 0 ($p<0.05$) but not signifant difference as comparable with group 1. BUN in hyperoxaluric rats (group 2-5) were increase after 21 days of treatment

($p$<0.05) when compared with baseline while BUN on day 21 in group 4 and 5 were higher than group 1 ($p$<0.05) (Table 4.17).

**Table 4.17** The levels of PCr (mg/dl) and BUN (mg/dl) in five groups of the study on day 0 and day 21.

| | period | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|---|
| PCr | Day 0 | 0.34±0.03 | 0.34±0.02 | 0.30±0.03 | 0.30±0.03 | 0.30±0.02 |
| | Day 21 | 0.44±0.04 | 0.53±0.06$^{\dagger}$ | 0.35±0.03 | 0.42±0.03$^{\dagger}$ | 0.41±0.04$^{\dagger}$ |
| BUN | Day 0 | 18.8±1.8 | 20.1±0.9 | 18.8±0.8 | 20.4±1.0 | 19.8±1.0 |
| | Day 21 | 20.1±1.1 | 25.8±0.8$^{\dagger}$ | 25.3±1.7$^{\dagger}$ | 29.3±2.4$^{*,\dagger}$ | 27.0±2.2$^{*,\dagger}$ |

The data are shown as mean±SEM.

$^{*}$ means significant difference when compared with group 1 ($p$<0.05)

$^{\dagger}$ means significant difference when compared with day 0 ($p$<.05)

### *Renal handling of organic and inorganic substances*

The urinary excretions and fractional excretions of Cl, Ca and Mg was unchanged among each group (Table 4.18). The urinary excretion of Na was highest in group 4, eventhough FE was unchanged. The urinary excretion and fractional excretion of K in group 2 and group 4 were significantly higher than group 1, while Vit E supplement (group 3 and 5) appeared to alleviate renal excretion of K (Table 4.18). Group 2 had significantly higher in either urinary excretion of Zn, V and FE of Zn ($p$ < 0.05) while Vit E or Vit C administration reduced renal excretion of Zn (group 3-5). The similar results were observed in excretion of oxalate while the result of citrate excretion was not changed. The risk indices including the urinary Ca/Mg, oxalate/ citrate

or Tiselius index were the highest in group 2 while $C_{Osm}$ and $C_{H2O}$ were unchanged among groups.

**Table 4.18** Effects of vitamin C and E and its combination on urinary excretion of organic (μmol/day) and inorganic (μEq/day) substances, FE of urinary electrolytes, Osmolar clearance (μl/day) and free water clearance (μl/day)

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| $U_{Na}V$ | 1810±241 | 2851±135 | 2703±405 | 3187±373* | 2466±369 |
| FE Na | 0.8±0.1 | 1.3±0.2 | 0.8±0.2 | 1.2±0.3 | 0.9±0.2 |
| $U_{K}V$ | 948±126 | 1582±101* | 1324±208 | 1622±149* | 1314±202 |
| FE K | 10.4±1.6 | 19.4±3.7* | 9.8±2.3# | 17.2±3.4* | 12.2±2.4 |
| $U_{Cl}V$ | 259±29 | 288±21 | 440±99 | 327±53 | 278±53 |
| FE Cl | 0.17±0.02 | 0.20±0.02 | 0.18±0.03 | 0.16±0.03 | 0.12±0.02 |
| $U_{Ca}V$ | 7.6±1.6 | 10.5±3.6 | 7.8±2.9 | 11.0±3.7 | 17.5±5.2 |
| FE Ca | 0.17±0.02 | 0.32±0.09 | 0.23±0.13 | 0.23±0.09 | 0.41±0.16 |
| $U_{Mg}V$ | 37.0±13.6 | 66.7±28.0 | 46.5±13.4 | 79.8±21.1 | 25.2±9.2 |
| FE Mg | 1.9±0.6 | 4.9±1.6 | 2.4±0.8 | 4.5±1.1 | 3.2±1.2 |
| $U_{Zn}V$ | 3.9±1.0 | 12.5±2.8* | 2.4±0.6 | 6.7±2.3 | 4.9±1.6 |
| FE Zn | 0.7±0.1 | 20.0±10.8* | 3.5±1.2 | 4.1±1.6 | 1.7±0.5 |
| Ca/Mg | 2.1±1.5 | 4.0±3.3 | 2.7±2.1 | 0.6±0.3 | 1.4±0.6 |
| Oxalate | 10.2±4.3 | 89.1±13.1* | 18.7±5.6# | 45.0±16.8# | 20.5±5.0# |
| Citrate | 28.1±7.5 | 42.3±13.8 | 22.0±7.3 | 22.8±4.1 | 72.2±21.3 |
| Ox/Cit | 0.6±0.2 | 3.3±0.8* | 1.1±0.4 | 2.1±0.7 | 0.3±0.1# |
| Tiselius index | 3.8±1.3 | 6.1±1.6 | 2.4±1.5 | 2.1±0.7 | 3.7±1.0 |
| $C_{Osm}$ | 21±3 | 38±3 | 44±8 | 32±3 | 38±9 |
| $C_{H2O}$ | -11±1 | -14±2 | -21±7 | -10±1 | -23±7 |

The data are shown as mean±SEM.

* means significant difference when compared with group 1 ($p<0.05$)

# means significant difference when compared with group 2 ($p<0.05$)

## C. Urinalysis results

The data from urinalysis revealed that hyperoxaluric groups (group 2-5) produced both COM and COD crystal in urine after 21 days of experiment while Vit E supplement (group 3) ameliorated COM score (Table 4.19). There was no change in urinay pH and urine specific gravity (USG) in each group. The urinary excretion of protein and urine volume were significantly elevated in group 2, 3 and 4 but in group 5 was increased without significant difference (Table 4.19).


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**Table 4.19** Excretion of urine protein (mg/day), urine volume (ml/day) urine pH, USG and crystal score from urinalysis at day 20.

| | group 1 | group 2 | group 3 | group 4 | group 5 |
|---|---|---|---|---|---|
| Protein excretion | 44±9 | 109±8* | 103±20* | 101±10* | 76±14 |
| 24 hr urine volume | 14.76±3.33 | 33.92±5.21* | 33.46±4.49* | 31.43±3.32* | 21.87±4.58 |
| Urine pH | 7.4±0.4 | 6.6±0.2 | 7.0±0.3 | 6.7±0.2 | 7.1±0.3 |
| USG | 1.021±0.003 | 1.021±0.001 | 1.029±0.004 | 1.025±0.003 | 1.022±0.002 |
| COM | 0.000±0.000 | 1.714±0.184* | 0.667±0.211# | 1.333±0.211* | 1.000±0.309* |
| COD | 0.000±0.000 | 2.000±0.218* | 1.333±0.211* | 1.833±0.167* | 1.429±0.202* |

The data are shown as mean±SEM

* means significant difference when compared with group 1 ($p<0.05$)

# means significant difference when compared with group 2 ($p<0.05$)

## D. oxidative stress parameters in urine, plasma and kidney tissues

The total antioxidant status in urine of group 2 was drastically reduced ($p<0.05$) when compared with control, but it was improved after receiving anti-oxidant (group 3-5) (Table 4.20). The MDA levels in plasma, urine and kidney tissues were higher in group 2 (Table 4.20, Table 4.21 and Figure 4-22) while vit E or vit C improved PMDA but not kidney MDA in group 3-5. The excretion of MDA in urine of hyperoxaluric groups (group 2-5) were increased with no significant difference among groups. The activity of SOD in kidney tissue was significantly increased in group 2 ($p<0.05$) with reduction in

reduced GSH. Giving vit E and vit C reversed SOD activity in all groups but not GSH levels except in combination group. No changes in CAT activity was found among groups.

**Table 4.20** Effects of Vit E and Vit C and its combination on PMDA (nmol/ml), UMDA/UCr (nmol/ mg Cr) and UTAS (%)

| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| PMDA | 2.0±0.2 | 3.8±0.2* | 2.2±0.4# | 2.1±0.6# | 2.4±0.4# |
| UMDA/UCr | 12.7±1.7 | 24.5±4.7 | 22.1±2.6 | 23.0±3.7 | 19.1±3.7 |
| UTAS | 84±1 | 34±3* | 72±6# | 78±3# | 75±3# |

The data are shown as mean±SEM

* means significant difference when compared with group 1 ($p<0.05$)

# means significant difference when compared with group 2 ($p<0.05$)

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**Table 4.21** The kidney oxidative status parameters including MDA (nmol/mg kidney protein), SOD activity (unit/mg kidney protein), CAT activity (unit/mg kidney protein) and reduced GSH (nmol/mg kidney protein) in five groups of the study.

| | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| MDA | 0.84±0.10 | 1.60±0.17* | 1.24±0.18 | 1.55±0.13* | 1.39±0.08* |
| SOD activity | 4.0±0.7 | 17.8±2.7* | 4.7±0.9# | 5.1±1.8# | 3.6±0.6# |
| CAT activity | 336±23 | 337±29 | 328±19 | 299±15 | 298±7 |
| reduced GSH | 4.12±0.43 | 1.87±0.24* | 2.19±0.15* | 1.95±0.17* | 3.00±0.23# |

The data were shown as mean±SEM

* means significant difference when compared with group 1 ($p$<0.05)

# means significant difference when compared with group 2 ($p$<0.05)

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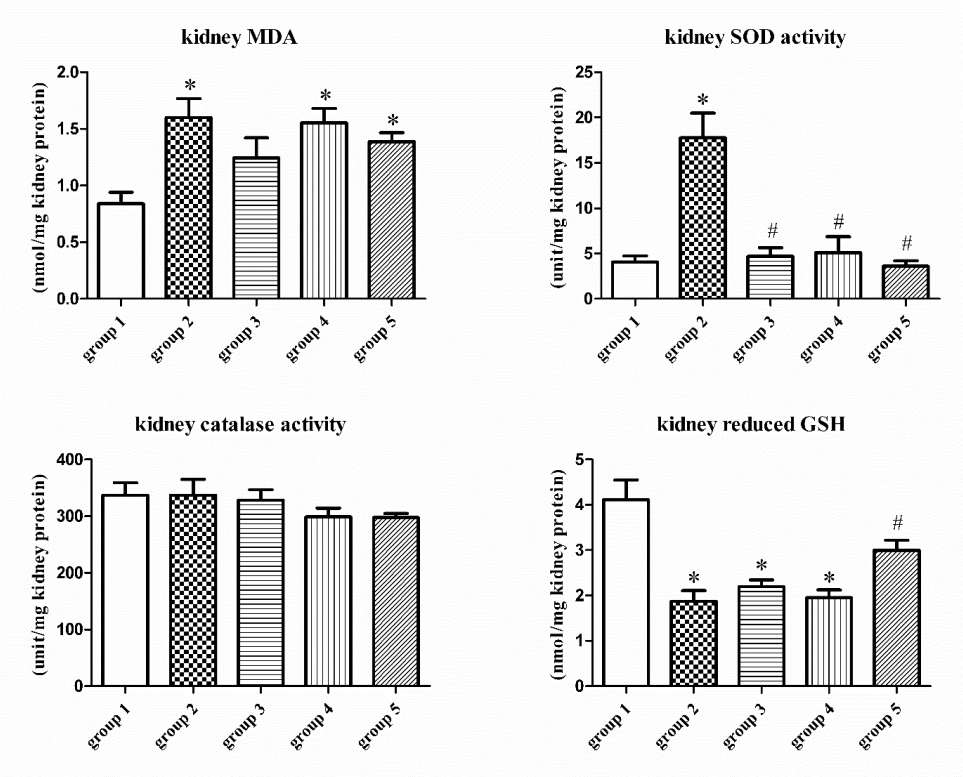


**Figure 4-22** The oxidative stress parameters in the kidney tissues were shown including the levels of MDA and reduced GSH and the activity of SOD and catalase enzyme. * means significant difference when compared with group 1 ($p$<0.05) # means significant difference when compared with group 2 ($p$<0.05).

## E. Soluble Klotho protein in urine and plasma

The levels of soluble Klotho protein were drastically reduced in group 2 when compared with the other groups (Table 4.22). However, the urine soluble Klotho/Ucr ratio was lower in group 2-4 but not significant difference. The combination of Vit E and Vit C increased soluble Klotho in urine significantly ($p$<0.05, Table 4.22).

**Table 4.22** Effects of vitamin C and E and its combination on soluble Klotho protein in plasma (ng/ml) and urine (ng/mg Cr).

| Soluble Klotho | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|
| Plasma Klotho | 5.0±2.2 | 1.1±0.2* | 5.7±2.1 | 1.4±0.2 | 3.2±1.3 |
| Urine Klotho/UCr | 0.6±0.1 | 0.4±0.1 | 0.6±0.1 | 1.0±0.3 | 4.5±1.9# |

The data were shown as mean±SEM

* means significant difference when compared with group 1 ($p<0.05$)

# means significant difference when compared with group 2 ($p<0.05$)

## F. Histopathology and Immunohistochemistry results

### *Histopathology results*

The histopathologic pictures of the kidney tissues in cortex and medulla in all groups were revealed in Figure 4-23 to 4-27. There was no remarkable lesion of glomerulus and tubular cells in both cortex and medulla in control group as shown in Figure 4-24. However, group 2 developed moderate to severe flattened tubular epithelial cells in proximal, distal and collecting duct regions (Figure 4-24). Antioxidant treatment appeared to alleviate glomerular and tubular injury, as milder tubular epithelial lesions were observed (Figure 4-25 to 4-27). The quantitative of lesion score in tubular cells were observed in Figure 4-28.

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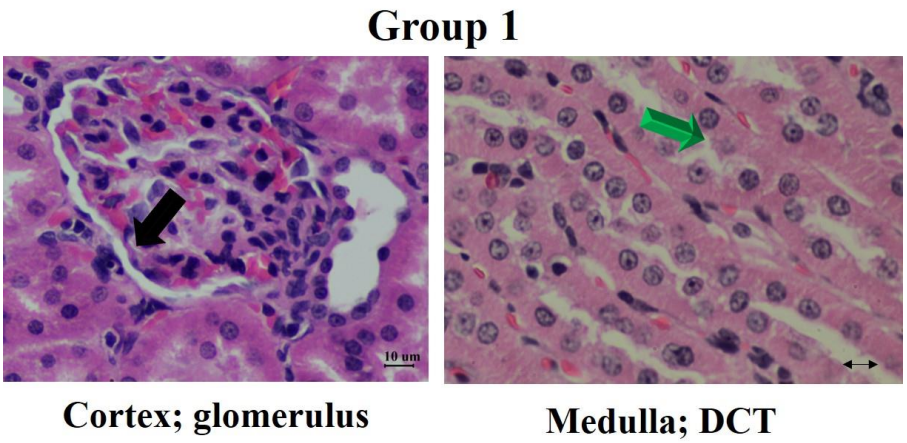


**Figure 4-23** Histopathology pictures of kidney tissue in group 1, no remarkable lesions of both glomerulus and tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space; a green arrow presented DCT in the medulla (x40, scale bar 10μm).

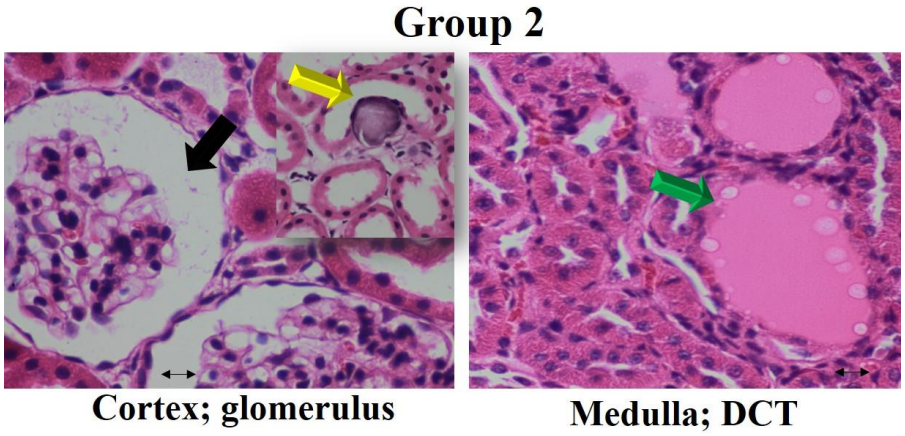


**Figure 4-24** Histopathology pictures of kidney tissue in group 2, mild lesions of glomerulus and moderate to severe lesions of tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space dilation; a yellow and green arrow presented crystal deposition and tubular cast in DCT in the cortex and medulla, respectively with marked flatted tubular epithelium and dilated lumen. (x40, scale bar 10μm).

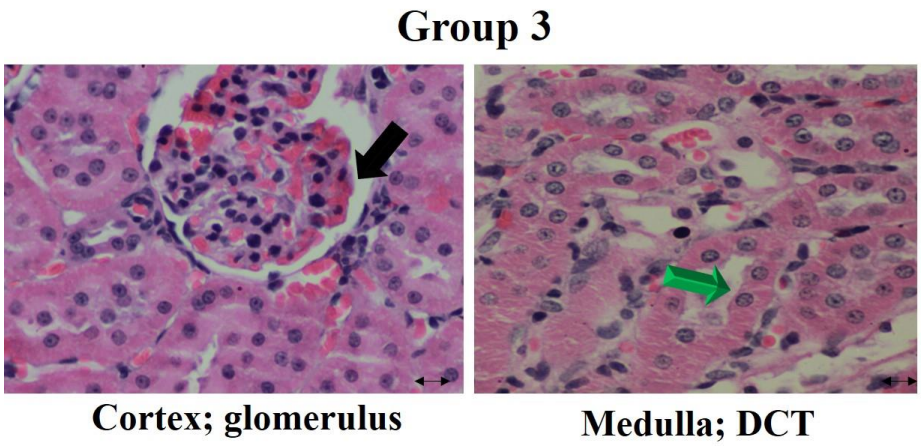


**Figure 4-25** Histopathology pictures of kidney tissue in group 3, no remarkable lesions of both glomerulus and tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space; a green arrow presented distal convoluted tubule in medulla (x40, scale bar 10μm).

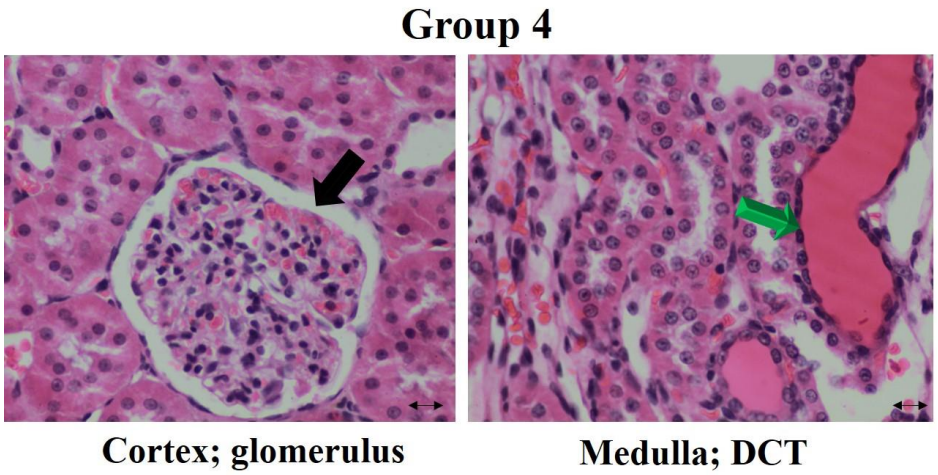


**Figure 4-26** Histopathology pictures of kidney tissue in group 4, no remarkable lesions in glomerulus and mild tubules lesions were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space; a green arrow presented distal convoluted tubule with protein casts in medulla (x40, scale bar 10μm).

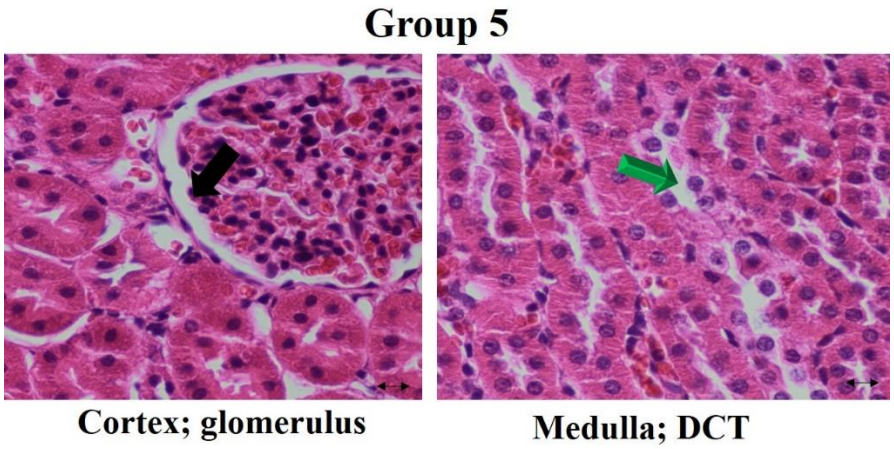


**Figure 4-27** Histopathology pictures of kidney tissue in group 5, no remarkable lesions of both glomerulus and tubules were noted; glomerulus in the cortex and black arrow presented Bowman's capsule space; a green arrow presented distal convoluted tubule in medulla (x40, scale bar 10μm).

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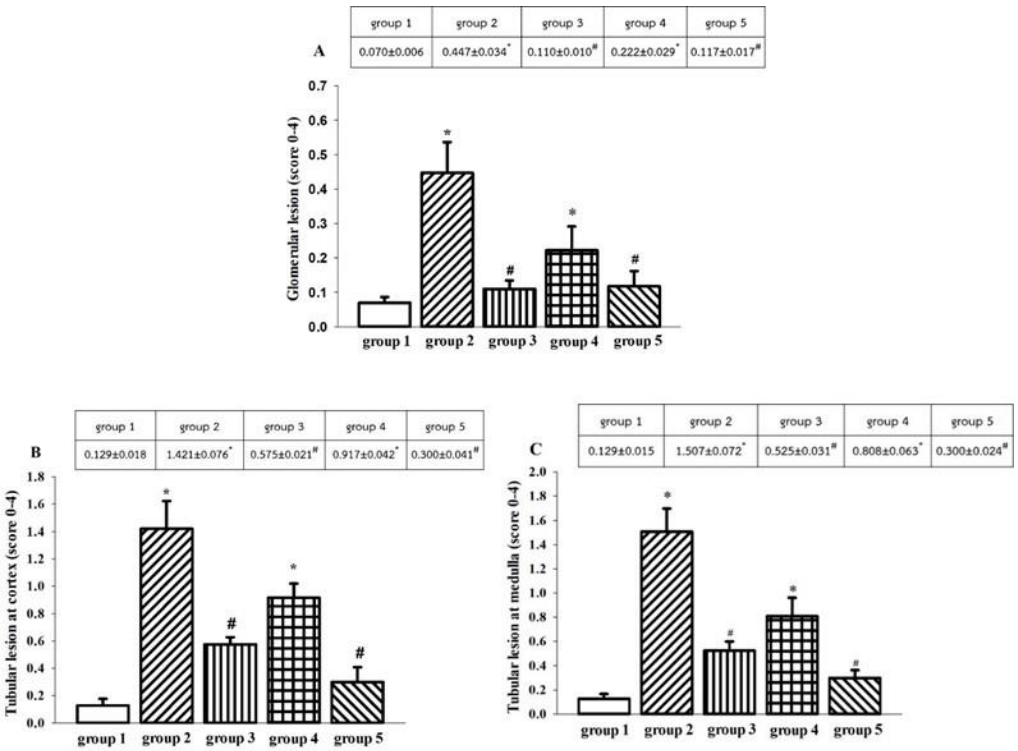


Figure 4-28 The severity score of 50 glomeruli were assessed and averaged (A) while the average of tubular lesions in cortex and medulla of kidney tissue were evaluated as shown in (B) and (C). Data were shown as mean±SEM. *$p$<0.05 compared with group 1, $^{\#}p$<0.05 compared with group 2.

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### *Immunohistochemistry results of kidney Klotho protein*

The Klotho protein in cortex and medulla of kidney tissues in each group were revealed in Figure 4-29 to 4-33 and quantitative data of positive area in all groups was represented. In the present study, the expression of membrane Klotho protein in either cortex or medulla was found in distal convoluted tubule and collecting duct. The Klotho protein expression in group 2 was progressively reduced when compared with group 1 while Vit E, Vit C and its combination protected renal Klotho protein. The similar results were found in quantitative data of positive area in all group of the study (Figure 4-34).

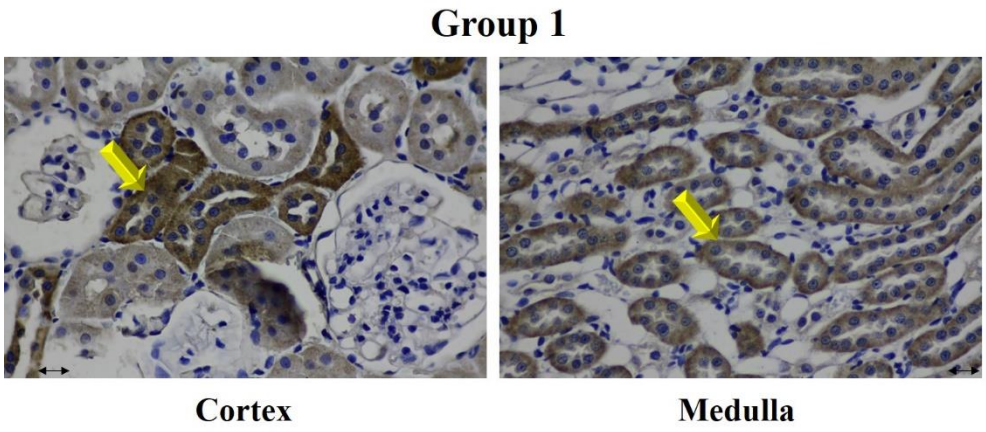


**Figure 4-29** Klotho protein expression in the kidney tissues at the end of the experiment of group 1 (x40 magnification, scale bar=10 μm). The yellow arrows represent positive area staining in cortex and medulla.

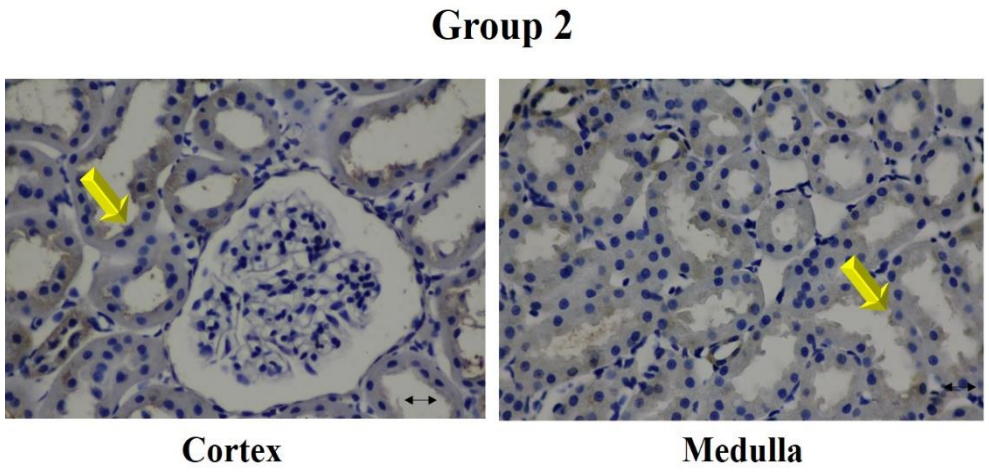


**Figure 4-30** Klotho protein expression in the kidney tissues at the end of the experiment of group 2 (x40 magnification, scale bar=10 μm). The yellow arrows represent distal tubule in cortex and medulla with negative results of kidney Klotho protein.

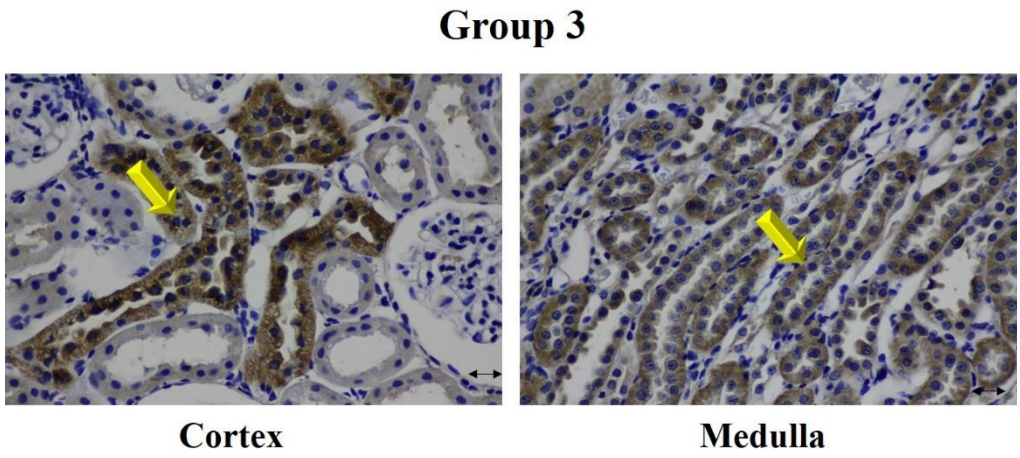


**Figure 4-31** Klotho protein expression in the kidney tissues at the end of the experiment of group 3 (x40 magnification, scale bar=10 μm). The yellow arrows represent positive area staining in cortex and medulla.

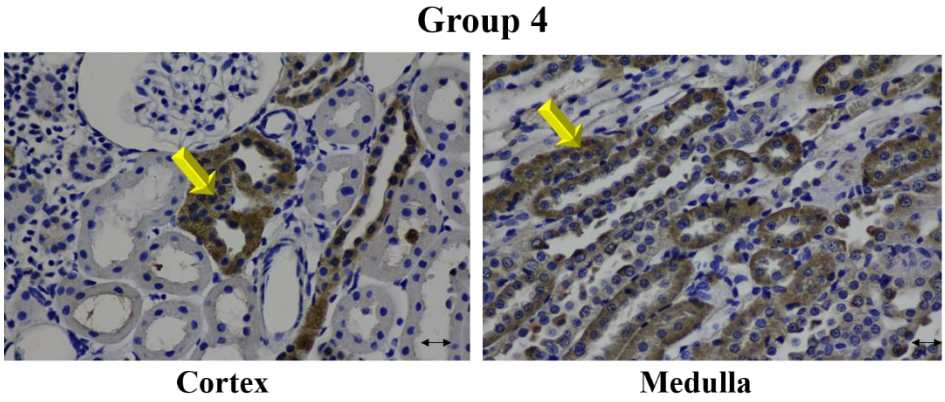


**Figure 4-32** Klotho protein expression in the kidney tissues at the end of the experiment of group 4 (x40 magnification, scale bar=10 μm). The yellow arrows represent positive area staining in cortex and medulla.

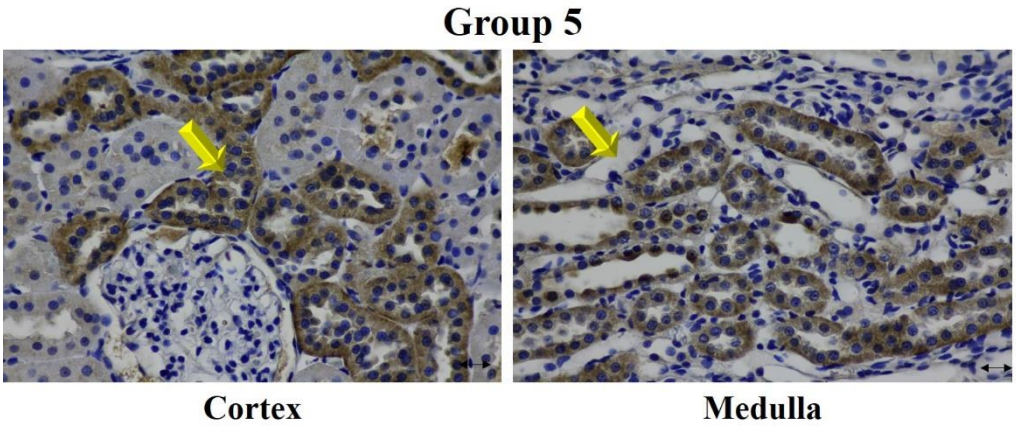


**Figure 4-33** Klotho protein expression in the kidney tissues of group 5 at the end of the experiment (x40 magnification, scale bar=10μm). The yellow arrows represent positive area staining in cortex and medulla.

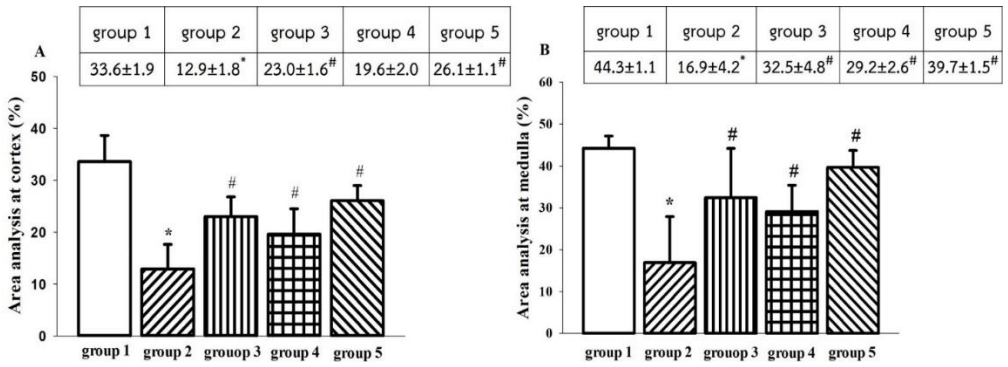


**Figure 4-34** The analysis of positive area (%) of kidney Klotho protein in cortex (A) and medulla (B) was evaluated by iSolution program (version 8.0). The data were shown as mean ± SEM. *$p$<0.05 compared with group 1, # $p$<0.05 compared with group 2.

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## G. The mRNA expression of *Klotho* gene by using real-time PCR

The mRNA expression of Klotho protein in group 2 was expressed at the higher level when compared with control group (Figure 4-35, Table 4.23). However, Klotho mRNA expression was unchanged in Vit C supplement rats (group 4) while group 3 and 5 showed higher expression of Klotho mRNA ($p<0.05$) compared with group 1. The data of the average threshold cycle of target gene (*klotho* gene) and reference gene (GAPDH) were presented in Table 4.23.

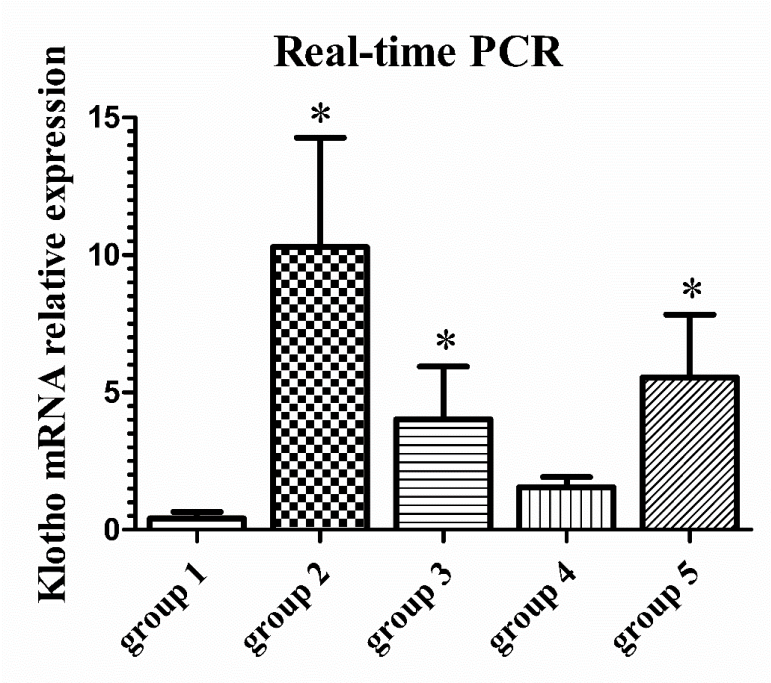


**Figure 4-35** The relative expression of klotho in five groups of rats. The picture shown the difference of mRNA expression in fold change. The calculation for the method of $2^{-\Delta\Delta_{CT}}$ as present in table 4.23.

**Table 4.23** The fold change expression of klotho after study, analyzed by $2^{-\Delta\Delta CT}$ method. Calculation from the difference of the average of $C_T$ of target gene and reference gene.

| Grouop | klotho average $C_T$ | GAPDH average $C_T$ | $\Delta C_T$ klotho-GAPDH | $\Delta\Delta C_T$ $\Delta C_T$ HLP treated - $\Delta C_T$ control | Fold difference in klotho, relative to control |
|---|---|---|---|---|---|
| Group 1 | 31.7±2.8 | 19.8±2.8 | 11.9±7.9 | 0.000±7.89 | 1 (232-0.004) |
| Group 2 | 26.6±1.4 | 17.6±0.8 | 9.0±1.4 | -2.9±1.4 | 7.5 (0.360-0.050) |
| Group 3 | 30.3±1.9 | 19.9±1.3 | 10.5±2.7 | -1.4±2.7 | 2.6 (2.523-0.059) |
| Group 4 | 33.8±2.3 | 21.8±2.2 | 11.6±5.1 | -0.4±5.1 | 1.3 (27.40-0.022) |
| Group 5 | 31.1±1.9 | 20.8±1.0 | 10.2±2.3 | -1.7±2.3 | 3.3 (1.507-0.062) |

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## H. The correlation between renal lesions score, Klotho protein and oxidative stress status

There was positive correlation between kidney reduced GSH and the % positive area analysis kidney Klotho protein (r= 0.608, $p$<0.01, n= 33) (Figure 4-36) while the negative correlation was found between % positive area analysis and kidney MDA and SOD activity (r=-0.617, $p$<0.01, n=33 and r=-0.565, $p$<0.01, n=33, respectively) (Figure 4-37 to 4-41). Moreover, the negative correlation was found in kidney Klotho and lesion score in cortex and medulla of kidney tissues (cortex, r= -0.813, $p$<0.001, n= 33 and medulla, r=-0.763, $p$<0.001, n=33, respectively) (Figure 4-42 to 4-43).

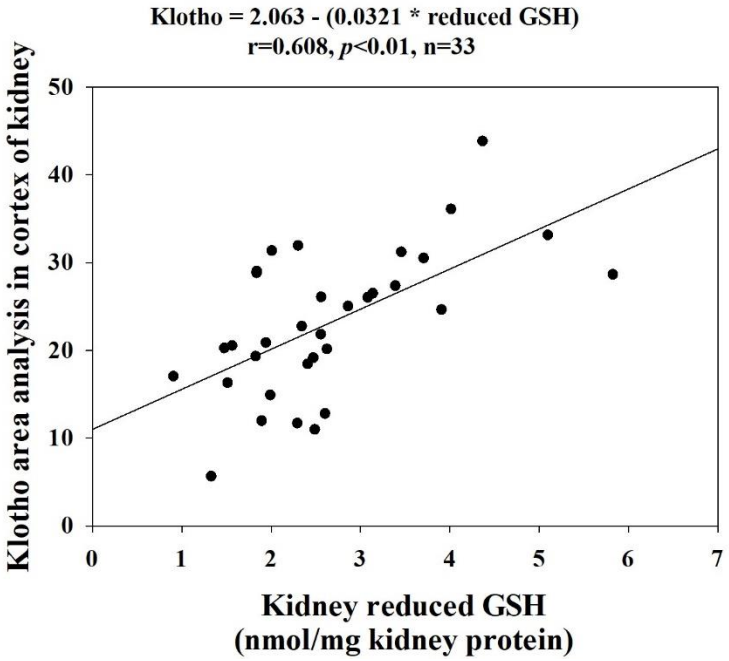


**Figure 4-36** The positive correlation and linear regression between kidney Klotho in cortex and reduced GSH.

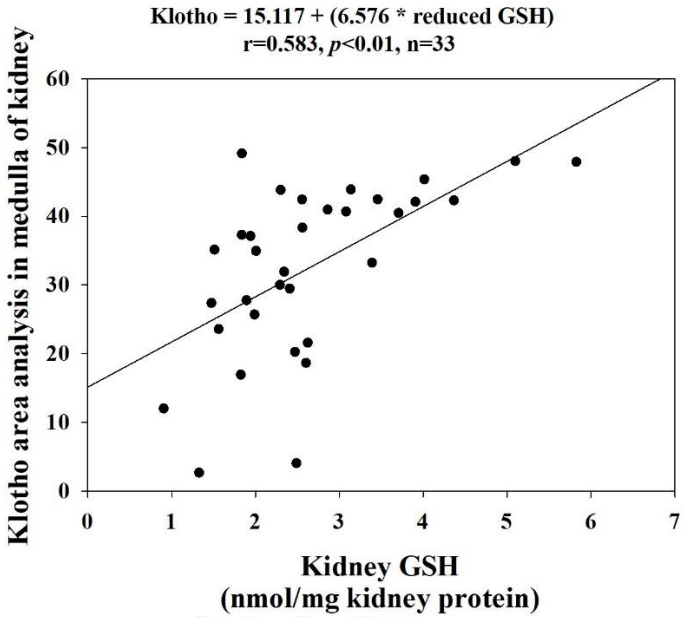


**Figure 4-37** The positive correlation and linear regression between kidney Klotho in medulla and reduced GSH.

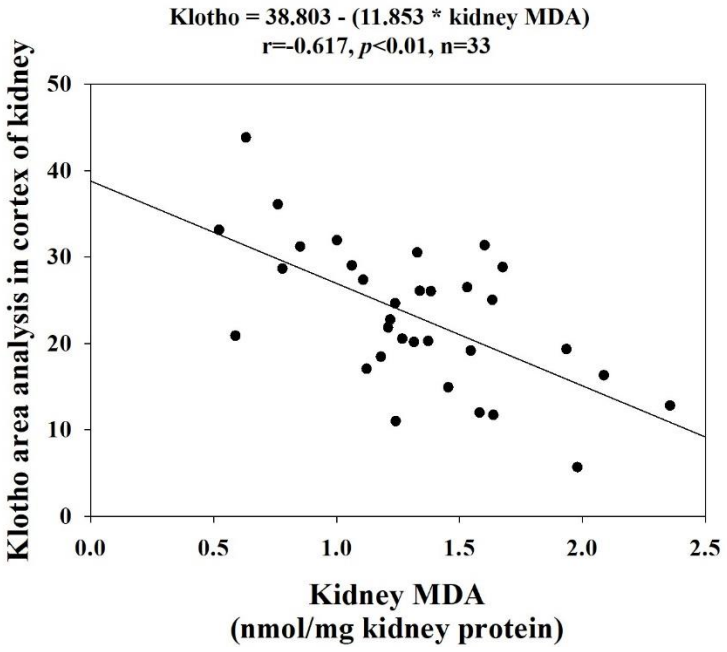


**Figure 4-38** The negative correlation and linear regression between kidney Klotho in cortex and MDA.

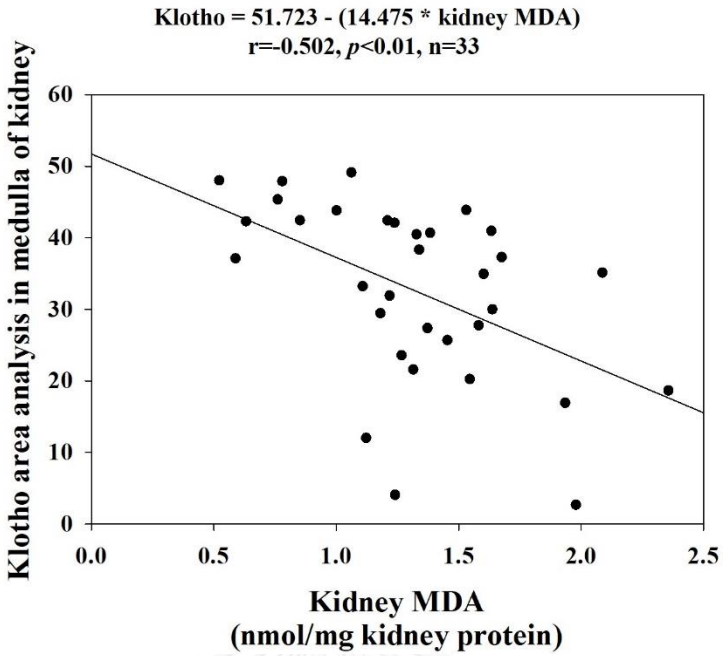


**Figure 4-39** The negative correlation and linear regression between kidney Klotho in medulla and MDA.

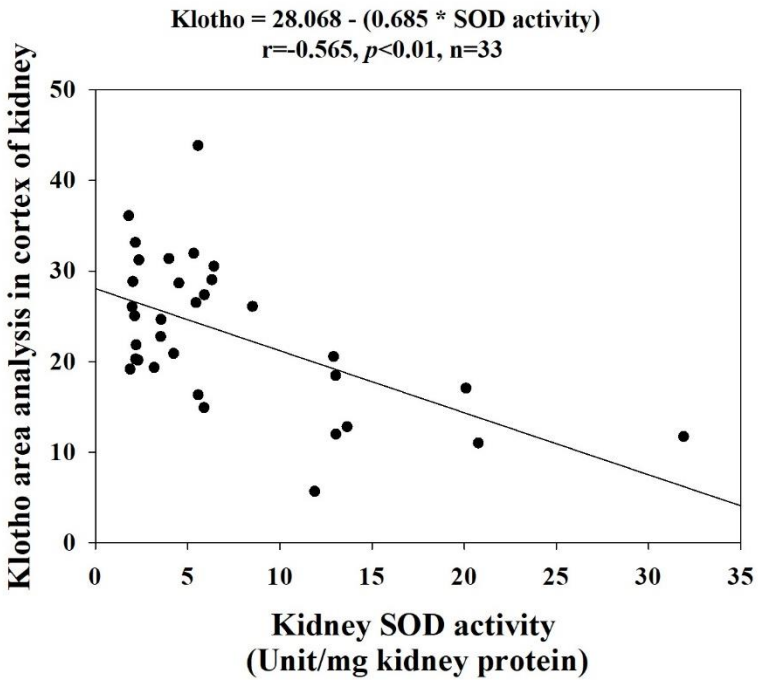


**Figure 4-40** The negative correlation and linear regression between kidney Klotho in cortex and SOD activity.

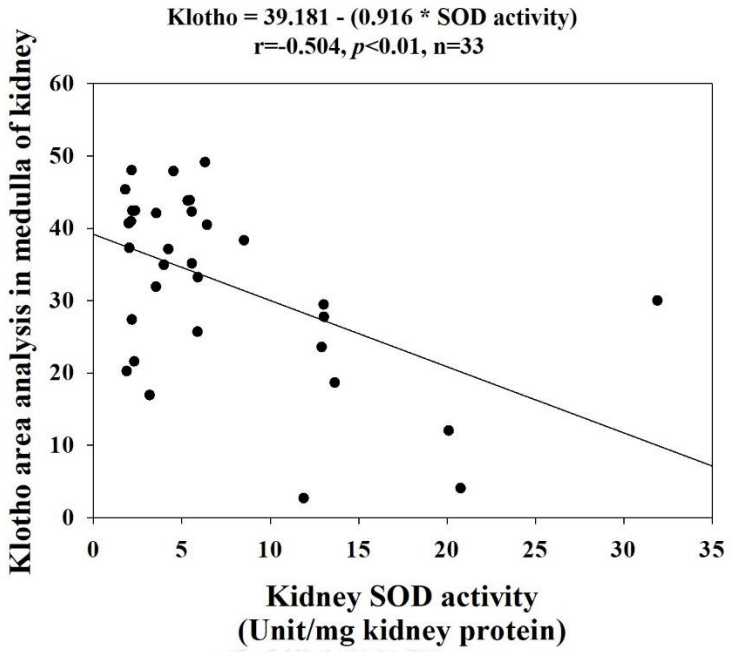


**Figure 4-41** The negative correlation and linear regression between kidney Klotho in medulla and SOD activity.

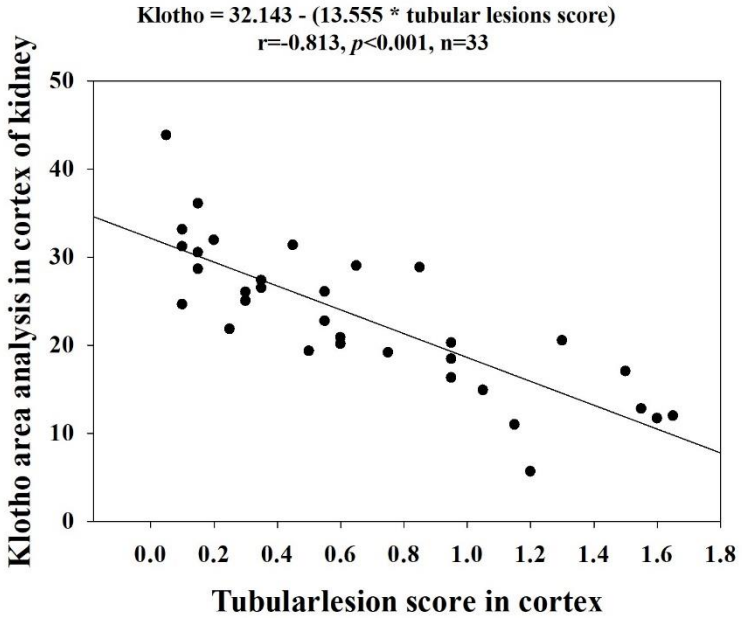


**Figure 4-42** The negative correlation and linear regression between kidney Klotho in cortex and tubular lesions score.

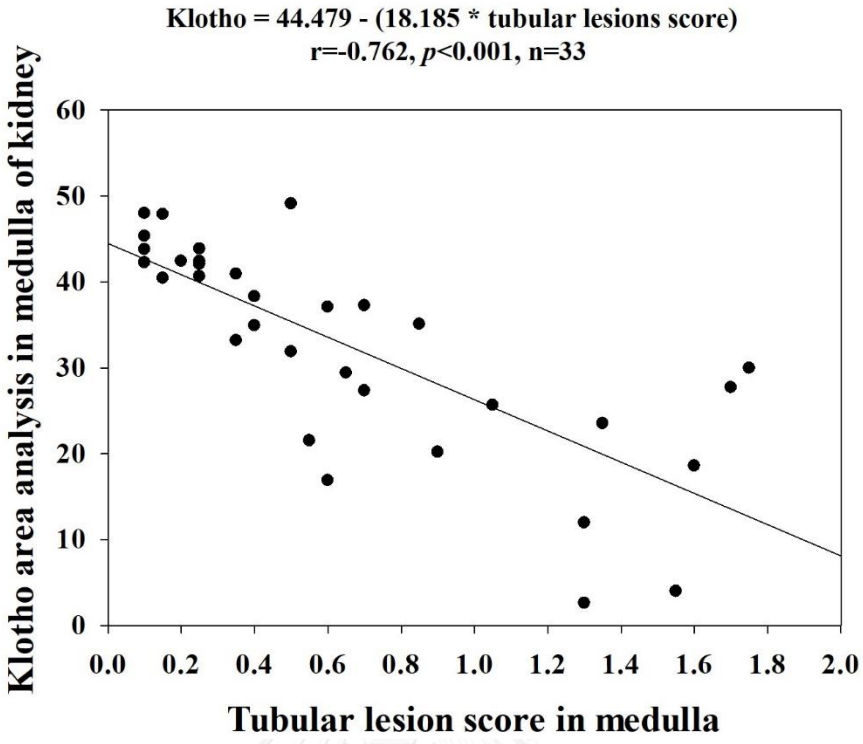


**Figure 4-43** The negative correlation and linear regression between kidney Klotho in medulla and tubular lesions score.

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# CHAPTER V

# DISCUSSION

***The study in part I, Protective effects of vitamin E and vitamin C supplement on renal functions, oxidative stress and renal handling of organic and inorganic substances in HLP-induced hyperoxaluric rats.***

In the present study, body weight was not different among groups. The food intake was slightly lower in group 3 at the end of the study. Moreover, water intake in group 2 was significantly elevated when compared with group 1, 3 and 5 only at the middle period of the study (day 10) while there was unaltered at day 21.

The general parameters of well-being of all experimental rats were changed within normal range, Moreover, the changes of PCr or BUN were shown in normal biological value as comparable with standard range. It could be due to the subclinical of acute phase hyperoxaluria induced nephropathy which was proved by the 50% reduction of GFR in HLP-treated rats (group 2). Moreover, in EG- induced hyperoxaluric rats and supplement with intraperitoneal injection of Vit E (twice a week), the body weight, was unaltered when compared among groups (Huang et al., 2006).

Lower body weight was found when using 0.8% EG in combination with 1% ammonium chloride ($NH_4Cl$) in drinking water giving in rats for 9 days' duration (Yamaguchi et al., 2005). In HLP induced hyperoxaluria model, decrease in food intake

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and body weight starting from day 7 and then throughout 42 days of experiment were demonstrated when using higher dose of HLP (5%) giving daily in the rat diet (Khan et al., 2006). On the contrary, the previous study using EG-induced hyperoxaluria in combination with low Vit E diet found that hyperoxaluric rats had low body weight but it was prevented in Vit E supplement group (Thamilselvan and Menon, 2005). Therefore, changes in body weight, food intake and water intake depend on dose and duration of both drugs induced hyperoxaluria and antioxidants.

The BUN and PCr in HLP-treated group tended to be higher than the other groups. These finding were consistent with a previous study (Thamilselvan and Menon, 2005). Moreover, the previous study found that creatinine clearance in hyperoxaluric rats was lower when compared with control rat (Khan et al., 2006; Yamaguchi et al., 2005). In addition, our study revealed that the supplement of Vit E, Vit C or their combination could ameliorate the levels of PCr and BUN as seen in EG-treated rat received excess Vit E in food. Hence, the administration of antioxidants in the present study, in separation or in combination, trended to recover both PCr and BUN in HLP-treated rats (group 3-5). The renal protective property of Vit E combined with Vit C was also shown by stabilizing BUN and creatinine found using multi-doses of Vit C and E (250 mg/kg each) in cisplatin model, in wich the renal GSH and MDA were also reversed (Ajith et al., 2009).

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Our results showed that the levels of plasma electrolytes were unaltered throughout the study period while plasma oxalate was elevated in group 2, 3 and 4. However, there are limited data of plasma electrolytes concentrations in the similar model.

Our results showed that the levels of plasma electrolytes were unaltered throughout the study, except plasma oxalate that was elevated in group 2, 3 and 4 compared with baseline. We assumed that the supplement of Vit E and Vit C had no effect on plasma electrolytes, and HLP supplement resulted in exclusively increased in plasma oxalate level.

The GFR in group 2 was significantly lower than control group suggesting that hyperoxaluria impaired the filtration process. Reduction in renal plasma flow was seen with less dosage compare to GFR resulting in slightly lower FF. In addition, the RVR was elevated in group 2 but not significant when compared with the other groups. Reduction in GFR in HLP induced hyperoxaluric rat could be due to the increased intra-capsular hydrostatic pressure from CaOx crystal tubular obstruction.

Using 5% HLP induced hyperoxaluria in rats stimulated crystals deposition in cortex, medulla and papillary tip which can be detected under polarized light microscope and scanning electron microscope after 28 days (Khan et al., 2006). Reduction in GFR, RBF and cortical microvascular blood flow were detected in model

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of EG giving at the dose of 0.75% in drinking water for 42 days along with oxalate infusion (Huang et al., 2003).

Besides, mechanical obstruction and kidney damage, oxalate, CaOx and calcium phosphate crystals could activate renal angiotensin II (A II) leading to the increased formation of reactive oxygen species and inflammation (Khan, 2013; Khan, 2014). Angiotensin II receptor antagonist was proved to be beneficial in rats induced hyperoxaluria using EG. The hyperoxaluria-associated renal lesions were including the crystal formation in tubular fluid, inflammation reaction and oxidative stress were reduced (Toblli et al., 2002). The transforming growth factor-beta (TGF-β) level in kidney tissue was not increased compared with stone forming rats (Yoshioka et al., 2011). Blocking AII receptor could also reduce osteopontin (OPN) expression along with reduced MDA and calcium crystal deposition in hyperoxaluric rats (Umekawa et al., 2004). Whether AII affects the renal tubular cotransport or other stone forming inhibitor need further elucidation.

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Although renal function did not return to normal, but our study indicated that Vit E could reduce the RVR, which suggesting that the mechanism may involve vasodilatatory effect. Previous report demonstrated that Vit E giving along with erythropoietin and iron could increase renal blood flow in gentamicin induced renal injury (Thongchai et al., 2008). Vitamin E could also alter vascular reactivity in chronic

bile duct-ligated rats (Alcaraz et al., 2007). Vitamin E also modulated the bioavailability of nitric oxide (NO) resulting in vasodilation (Green et al., 1998).

In the present study, improved GFR and ERPF with reduction in BUN in group 4 and 5 may provide a plausible linking mechanism that Vit C involved the restoration of NO activity after oxidative stress which was demonstrated in essential hypertensive patients (Taddei et al., 1998). Some actions of Vit C to improve defective endothelial-dependent vasodilation via nitric oxide were reviewed (May, 2000).

In model of calcium oxalate nephropathy, Vit E or Vit C alone provided protection against oxalate induced cell injury and restore antioxidant status when study in LLC-PK1 cells (Thamilselvan et al., 2014) although Vit C could not protect in one study of culture renal epithelial cells (Fishman et al., 2013). The remarkable protection was found when giving combination as was seen in the present study. Group 5 had the highest GFR which reverse near normal level as comparable with group 1. It is interesting that using combination of Vit E and C may be more advantage than giving alone. Improved GFR, renal plasma flow and reduced tubular cell injury as well as reduced glomerular necrosis, proteinuria and blood pressure were found in Dahl salt-sensitive rats giving high sodium and received 111 IU/day Vit E in food and 98 mg/day Vit C in drinking water for 5 weeks (Tian et al., 2005). These responses were corresponding to the decline in renal cortical and medullary superoxide anion release. Another study in rats showed Vit C (2500 unit/kg) and Vit E (1000 unit/kg) giving in

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combination could amiloriate acute nephrotoxicity induced by cisplatin (Atasayar et al., 2009).

Not only impairment of glomerular filtration, the tubular damage was also affected by oxalate. The new evidences were increasing related to the role of proximal tubule as primary sensor and effector in the progression of AKI and CKD (Chevalier, 2016). The tubular dilation and loss of epithelial cells with tubular regeneration were found in this model (Khan et al., 2006). The changes are mostly limited the capacity of tubular cells in which the crystal was deposited. This is the first study demonstrated that hyperoxaluria decreased tubular reabsoption of both Na and water at the proximal but not the distal tubule. The degrees of impairment of Na and water were similar which confirmed the presence of iso-osmotic transport at this site. The renal handling of oxalate was mainly excreted at proximal tubule using solute carrier family 26 member 6 (SLC26A6), an oxalate transporter (Ohana et al., 2013). Oxalate could enter the tubular lumen by Na-dependent transport process. A higher filter load of oxalate was responsible for higher excretion in group 2. Therefore, oxalate itself may damage the tubular membrane and limit the transport of other compounds as seen in reduction of Na and water reabsoption. Nevertheles, one report using EG with oxalate infusion yielded the different results in which 42 days of administration resulting in reduction in urine flow and Na excretion (Huang et al., 2003). The differences in results may be due to different model of hyperoxaluria.

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Compared with the distal tubule, the proximal tubule is more susceptible for injury caused by ischemia, oxidative stress or toxin due to low anaerobic glycolysis (Chevalier, 2016). Moreover, this site is lack of antioxidant and antiapoptotic proteins (Kiyama et al., 1995). Previous study demonstrated that distal tubule can secrete factors and cross talk with proximal tubule to enhanced cell survival after injury (Gobe and Johnson, 2007). The results suggest that the proximal tubular part of the nephron is the site of action of oxalate. Unlike this model, giving intra-abdominal administration of glycoxylate 100 mg/kg/day in mice showed crystal deposition in renal distal tubular lumen with lumen dilatation (Hirose et al., 2010).

Supplement with vitamin C in HLP-treated rat rescued the capacity of Na and water tubular reabsorptions at proximal tubule to nearly normal.

In this present study, the damage of proximal tubule was rescued by administered of vitamin E, vitamin C and especially the combination of vitamin E and C. The interplay between vitamin E and vitamin C may increase potential effect of ROS scavenger which proved by some previous study (Niki et al., 1985; Kadkhodaee et al., 2005).

The citrate excretion was reduced in this study along with reduction in plasma citrate. Report of increased citrate excretion after vitamin E treatment was found after 60 days of treatment and was suggested to reduce the retention of calcium oxalate stone in hyperoxaluria patients (Anbazhagan et al., 1999). Moreover, vitamin E could

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normalize biochemical alterations and restored kinetic properties of Tamm-Harsfall glycoprotein which promoted nucleation and aggregation phases of stone found in hypertensive and hyperoxaluric patients (Sumitra et al., 2005). Another inhibitor for CaOx stone formation is Mg (Riley et al., 2013). In the present study, however, vitamin E had no effect on Mg excretion while CaOx crystal formation was reduced. Thus, Mg may not play a crucial role for stone formation in this model.

Previous study showed higher COM crystal and COD stone when 5% HLP was given in food along with higher water intake and urine volume (Khan et al., 2006) similar to our results. Enhanced dosage was not related to concentrating ability of the final urine.

The urinary excretion of oxalate was increased in Gr. II as seen using EG model (Huang et al., 2006). Moreover, Ca excretion was slightly reduced after HLP treatment. The reduced Ca excretion although enhanced CaOx crystal was also similar to the previous study using 5% HPC in food which could be detected starting from day 7 until day 42 (Khan et al., 2006). This finding could be explained by calcium deposit in kidney tissue and may be precipitated in urine sediment. The formation of CaOx stone was then dependent on oxaluria rather than enhanced calcium excretion as suggested by Lee et al. (1992) who showed that high calcium in the diet or giving EG cannot induce CaOx stone without hyperoxaluria. The results suggested that even low calcium content in the urine can cause stone formation. Rather than oxalate, the urinary citrate

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also plays a key role to control stone formation. The combination of hyperoxaluria and hypocitraturia with absence of hypercalciuria could be found in Ca oxalate stone forming patients (Anbazhagan et al., 1999). The interplay between SLC26A6 and NaDC-1 transporters to determine oxalate and citrate homeostasis was proposed (Ohana et al., 2013).

Microscopic examination of urine sediment in our study revealed that hyperoxaluric rats supplement with vitamin E, vitamin C or its combination could reduce both COM and COD crystal. The study by Huang and colleagues (2006) revealed that administration of vitamin E protected crystal formation and accumulation in the kidney tissue by enhance the levels of anti-lithogenic molecules, osteopontin (OPN) and Tamm-Horsffall protein (THP) but not in urine sediment.

Moreover, strong correlation was found between oxidative stress and oxalate excretion. Giving EG in vitamin E deficient rats caused massive CaOx deposition with enhanced oxidative stress that could be reversed after supplement with vitamin E (Thamilselvan and Menon, 2005; Huang et al., 2009).

The association between ascorbate ingestion and stone formation were investigated in the previous studies (Chalmers et al., 1986; Gerster, 1997; Auer et al., 1998; Curhan et al., 1999). Loading ascorbic acid does not stimulate calcium oxalate supersaturation and crystallization in patient with CaOx uroltiasis (Schwille et al., 2000). In the present study, group 4 that received vitamin C had higher plasma oxalate. Thus,

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enhanced filter load of oxalate was expected. The COM and COD crystal formations in group 4 were lower than group 2 which received HLP alone suggesting no detrimental effect of vitamin C.

Recently, lime based trial drug has been used in nephrolithiasis patients in the northeastern part of Thailand and exerted citraturic and alkalinizing actions as efficient as potassium citrate (Tosukhowwong et al., 2007). It also had antioxidant effect and attenuated renal damage. Moreover, previous study showed that lemon juice plus anti-oxidant nutrients putting in diets could lower the number of crystal deposition in rats receiving EG resulting in renal protection and preventing stone deposition (Naghii et al., 2014). Although the effect of lime base will be focus on potassium citrate replacement, the action of vitamin C in lime base is also existed.

In the present study, increased PMDA with low UTAS in group 2 were found. However, the UMDA/UCr was unchanged. which may be mainly due to dramatic dropped in GFR. Although oxidative stress may play a key role in CaOx stone formation, Huang et al (2006) suggested that the causes of stone formation at the early stage may rather be due to changes in OPN and THP in kidney tissue and urine which responsible for nucleus formation. The LLC-PK1 cells exposed to oxalate showed increased in LDH release and MDA content which was further elevated when COM crystal was added (Thamilselvan et al., 2003). The mechanisms of oxalate induced peroxidative injury

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through the activation of NADPH oxidase via induction of TGF-β1 and GSH redox imbalances were demonstrated in culture cells (Rashed et al., 2004).

Giving vitamin E in the present study showed reduced oxidative stress as suggested by lower MDA and higher UTAS. The results were similar to report in rats receiving EG at 150 mg/kg by gavage for 3 weeks which showed that excess vitamin E could completely prevent the CaOx deposition in renal tissue along with the restoration of tissue antioxidants (Thamilselvan and Menon, 2005).

In culture LLC-PK1 cells, vitamin E reduced LDH release, restore antioxidant enzyme activities and reduced MDA when giving with either oxalate alone or in combination with COM crystals (Thamilselvan et al., 2003). The protective effect of vitamin E is superior than SOD, CAT and DFO. Vitamin E is also increased cell viability and reduced TGF-β1 expression which increased after oxalate exposure (Rashed et al., 2004).

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Report of hyperoxaluria rats induced by EG given for 42 days showed that vitamin E could not completely eradicate the oxidative stress as shown by higher urinary TBAR and MDA especially at 21 days (Haung et al., 2006). They proposed that the lower CaOx crystal in kidney tissue and urine sediment may primarily due to changes in defensive effects caused by OPN and THP. In the present study, group 3 had lower CaOx crystal and oxidative stress parameter compared with group 2 which

may in part due to antioxidant mechanism. However, the anti-oxalate or defense markers may be involved.

In human patients with hyperoxaluria, increased plasma lipid peroxide with decreased levels of vitamin E, vitamin C, blood GSH and hemolysate SOD and GPX were found (Anbazhagan et al., 1999). These effects were normalized nearly 100% after 90 days receiving 200 mg/day of vitamin E supplement. In the present study, the dose of vitamin E is far behind the basal dietary requirement in rats which is 3 IU/kg per day (Attia et al., 2001). Vitamin E could protect kidney when giving along with HLP as shown by higher GFR and lower BUN and Cr. Tubular reabsorption of Na and water were also improved. The COD and COM crystal formation was less compared with group 2 which received HLP alone.

HLP-treated rats supplement with vitamin C had lower both PMDA and crystal formation compared with group 2 suggested that vitamin C also protected the kidney from oxidative damage.

Vitamin C is a strong antioxidant which is water soluble. It can react as scarvenging superoxide, hydroxyl radicals and single oxygen. Vitamin C was proved to be effective in preventing renal damage with reduced renal histopathologic lesion and oxidative stress especially in model of ischemic reperfusion injury in nephrectomized rats (Korkmaz and Kolankaya, 2009). In I/R model, the renoprotective dose of vitamin C was seen at the dose of 250 mg/kg while dose up to 1000 mg/kg may cause higher

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lipid oxidation induced hepatic injury. The renoprotective effects of high dose of vitamin C were demonstrated in short term 24 hr study of gentamicin-induced nephrotoxicity (Moreira et al., 2014).

In oxalate nephropathy model, although the mechanism of renal injury involves oxidative stress, vitamin C usage was limited due its metabolite resulting in the formation of oxalate (Longenecker et al., 1940). Secondary hyperoxaluria was found in renal transplantation patients after excess vitamin C intake (Yaich et al., 2014).

Previous study reported that vitamin E and C in combination treatment could prevent increase in MDA in kidney, liver and erythrocyte. Moreover, BUN was not increased compared with giving cisplatin alone (Kadkhodaee et al., 2005). The prevention as shown by unaltered BUN and creatinine was also found using multi-doses of lower concentrations of vitamin E and vitamin C (250 mg/kg each) in cisplatin model whereas the renal GSH and MDA were also reversed (Ajith et al., 2009). Moreover, beneficial effects of co-supplementation of vitamin C and vitamin E were found in gentamicin induced nephrotoxicity in which GFR and GSH level were preserved while enhanced urinary enzyme activity was prevented (Kadkhodaee et al., 2005). Thus, combination may be useful in model of renal impairment mediated via oxidative stress damage.

Finally, the histopathology of kidney tissue confirmed that the lesion was mainly seen at the proximal tubule. No dramatic lesion was detected at the glomerular

part corresponding to less blood flow or high tubular pressure rather than pathology at glomerular filtration barrier. The tubular cells were swollen which were similar to the previous report in which the tubular cell injury with positive PCNA (proliferating cell nuclear antigen), an indicator of tubular cell regeneration after tubular epithelial loss was found (Khan, 2006). The same research group showed results from immunohistochemistry with upregulation of CD44 and OPN along with crystal retention and adhesion in renal tubule.

The histopathologic also confirmed that kidney of rats in group 5 had the lowest lesions compared with other groups. The mechanism in which combination of vitamin E and vitamin C is more advantage may be due to vitamin E was depleted dramatically after oxalate exposure. Giving vitamin C can help recycling and replenish the storage of vitamin E as suggested by Niki and co-workers (1985).

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The highly correlation between GFR and oxidative stress parameters (PMDA and UTAS) could be hypothesized that overwhelming of ROS from hyperoxaluria interrupted filtration process of the kidney and could be alleviated by using the synergistic effect of vitamin E and C.

Therefore, the maintaining of redox homeostasis could protect the kidney function from oxalate induced nephropathy.

***The study in part II, effects of vitamin E and vitamin C supplement on oxidative stress status, Klotho protein levels and Klotho protein mRNA expression in HLP induced hyperoxaluric rats.***

In this study, the body weight and food intake were unchanged with significantly increase of water intake and urine flow rate in hyperoxaluric group. It was hypothesized that there was subclinical impairment of kidney functions due to the dosage of HLP and duration of the experiment in our study compared with previous studies.

Plasma electrolytes concentration, organic and inorganic substances excretions were similar to the study in part I. The crystal formation was also similar to the study in part I. Additionally, the systolic blood pressure and plasma oxalate concentrations were drastically elevated in hyperoxaluric group. Hypertension is the one of many risk factors in kidney stone formation (Borghi et al., 1999) while increase in blood pressure after CaOx formation could be due to post-glomerular obstruction, crystal aggregation leading to tubuloglomerular feedback (TGF). The crystal in tubule of nephron could be directly obstructed or disturbed the flow of ultrafiltrate. Study by using computational simulation of renal function in the Randall's plugs and calcium oxalate crystalluria found that the plasma oxalate levels at 2.75 μmol/l influenced productivity of CaOx crystal precipitation at the end of the descending limb of the loop of Henle. Besides, the plasma oxalate at 1.4 μmol/l achieved the empirical thermodynamic

formation product leading to precipitation of CaOx in the collecting duct (Robertson, 2015). Importantly, the plasma oxalate levels in hyperoxaluric rats in our experiment exerted concentration over 200 umol/l. It's closely related in crystalluria in group 2. The TGF was activated for control of NaCl and volume in tubular compartment of the nephron resulting in secretion of adenosine and increased in calcium ion levels subsequently constriction of vascular smooth muscle in afferent arteriole.

Moreover, it was found that CaOx stone activated renin-angiotensin system resulting in over production of angiotensin type II (A II) and vasoconstriction subsequently increased blood pressure via NADPH oxidase (Umekawa et al., 2004; Tsuji et al., 2016). Many previous studies reviewed that angiotensin type 1 receptor blocker (AT1) administration in CaOx stone were alleviated CaOx crystal deposition in kidney tissue of EG induced CaOx rats' model (Yoshioka et al., 2011). The kidney lesions after supplement with candesartan (angiotensin II receptor blocker) were also improved (Umekawa et al., 2004). The CaOx crystals could activate inflammatory cytokines (Mulay et al., 2013) and RAAS system.

In addition to the results of PMDA and UTAS which were similar to the results in part I, the kidney oxidative stress status was determined.

The enzymatic antioxidant activity in the kidney tissue of the present study revelaed that kidney SOD was drastically elevated in contrary to the kidney reduced glutathione in hyperoxaluric rats while catalase activity was unchanged among groups.

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It might be implied that ROS, superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$), in hyperoxaluria were overwhelmed which scavenged by using SOD, and subsequently with catalase and glutathione peroxidase, respectively.

The vitamin E or alpha-tocopherol has been designated an important role in kidney injury and others redox imbalance disease for many years ago according to its highly potent free radical scavenger fat soluble vitamin which controlling many cellular signaling pathways. Plasma concentration of vitamin E and vitamin A dropped in urolithiasis patients was contrary to plasma malondialdehyde levels (Anbazhagan et al., 1999; Kato et al., 2007). Administration of vitamin E orally (200 mg daily for 90 days) could improve oxidative status.

However, our study found that supplement isolated of vitamin E could not restored a kidney reduced GSH levels but vitamin E treated group had near-normal kidney morphology. The study of hyperoxaluric rats induced by EG for 42 days' duration showed that vitamin E could not eliminate the oxidative stress as shown by higher urinary TBAR and MDA especially at 21 days (Huang et al., 2006).

Vitamin C, ascorbic acid, is a highly potent water-soluble antioxidant. Previous study demonstrated that vitamin C was highly effective agents for renal damage with reduced renal histopathologic lesion and oxidative stress especially in model of ischemic reperfusion injury (IRI) in nephrectomized rats (Kadkhodaee et al., 2005).

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In IRI model, the renoprotective of vitamin C was realized at the dose of 250 mg/kg while dose up to 1000 mg/kg could produce higher lipid oxidation with subsequent hepatic injury. The renoprotective effects of high dose of vitamin C at 1 g/kg/day by gavage were demonstrated in short term 24 hr study of gentamicin-induced nephrotoxicity (Moreira et al., 2014). On the contrary, it was reported that metabolic waste of vitamin C is oxalate. Using vitamin C for prevention of ROS in hyperoxaluria or CaOx stone was controversial. The association between ascorbate ingestion and stone formation were investigated in the previous studies (Chalmers et al., 1986; Curhan et al., 1997; Gerster, 1997; Auer et al., 1998).

Moreover, rats in group 4 showed the more balance redox homeostasis than rats in group 2 and improved both Klotho mRNA expression and protein levels with lower score lesion from pathologic study in the kidney tissue. It was implied that reno-protective role of vitamin C could be supported according to renal handling of oxalate and the other promoters and inhibitors of CaOx crystal formation.

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Unaltered BUN and creatinine was also found after using multi-doses of lower concentrations of vitamin C and E (250 mg/kg each) in cisplatin model whereas the renal GSH and MDA were also reversed (Ajith et al., 2009). Moreover, beneficial effects of co-supplement of vitamin C and E were found in gentamicin induced nephrotoxicity in which GFR and GSH level were preserved while enhanced urinary enzyme activity was prevented (Kadkhodaee et al., 2005). It was postulated that the combination may

be useful in model of renal impairment mediated via ROS scavenging mechanism. On the other hands, the levels of kidney reduced glutathione in group 5 of the present study were higher than group 3 and group 4 which could be due to the synergistic effects of Vit E and Vit C.

Study in oxalate induced oxidative stress in LLC-PK1 kidney cell line, combination of vitamin E and vitamin C was more remarkable protection than isolated-supplement (Thamilselvan et al., 2014). We confirmed from our results that vitamin E and C in combination had higher protective effects compared with giving in isolation especially urine oxalate excretion, risk indices, kidney reduced GSH, kidney SOD activity, Klotho protein levels (plasma, urine and kidney tissue) and kidney morphology.

Histopathology results demonstrated that the lesion was mainly seen at the proximal tubule. No dramatic lesion was detected at the glomerular part corresponding to less blood flow or high tubular pressure rather than pathology at glomerular filtration barrier similar to the study in part I. Moderate to severe of tubular cell flattening and tubular dilation were shown in group 2 and recovery after receveing Vit E and/or Vit C. The histopathologic also confirmed that kidney of rats in group 5 had the lowest lesions compared with other groups.

This is the first study to discover weather hyperoxaluria condition affected Klotho mRNA expression, protein levels and protein expression. We hypothesized that Klotho protein plays an important role in CaOx stone formation and recurrence. The

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overwhelming of ROS from hyperoxaluria affected Klotho protein and supplement of vitamin E could be completely protected soluble Klotho protein both in urine and plasma and kidney Klotho protein.

The protein expression of Klotho in the kidney tissues showed drastically reduction in group 2 and recovery after receveing Vit E and/or Vit C There are many factors involved protein levels including transcription rate, nuclear export, mRNA localization, transcript stability, translational regulation and protein degradation while protein activity may be affected by posttranslational modifications, glycosylation, nitrosylation, phosphorylation and proteolytic cleavage. Until now many studies revealed the direct and indirect effects of ROS damaged biomolecules by involving the structure, function, half-life or bioavailability. We now know that there are overproduction of ROS or toxic species in aging and senescence (Hamilton et al., 2001; Borel et al., 2013) and not only mutant klotho (Kuro-o et al., 1997) but also in hyperoxaluria and CaOx urolithiasis as shown by abnormality expression of Klotho gene. The damage and injury of kidney overproducted ROS which could be directly destroyed the Klotho protein not only soluble form in plasma and urine but also Klotho protein in kidney tissues. Moreover, the overwhelming of ROS could be affected indirectly to the half-life or structural change of the Klotho protein.

The mRNA expression showed contradiction results which was elevated in group 2. and reduced after supplement with Vit E and/or Vit C. The control of mRNA

expression could be denoted the important process of expression of the gene. There are many factors which plays a vital role in the difference mechanisms such as transcription control, RNA processing, RNA transport and localization, mRNA degradation or mRNA and protein interaction. Nevertheless, the expression of mRNA Klotho protein in kidney injury and impairment was inconsistent since it was reduced in IRI rat model while it was elevated in CDDP induced kidney injury (Kim et al., 2016). Moreover, Klotho mRNA expression were down regulated in CKD patient's due to suppression by uremic toxin (Sun et al., 2012).

It is evidence suggesting that mRNA expression and Klotho protein production might be disturbed by ROS-induced endoplasmic reticulum stress (ER stress) (Banerjee et al., 2013) and ROS-damaged renal tubular cells which is the productive source of Klotho protein (Kuro-o et al., 1997). Additionally, the feedback mechanism after reduction of Klotho protein levels for up-regulation of Klotho mRNA expression by using fibroblast growth factor receptor 23 (FGF23) could be diminished due to it was found that acute phase of inflammation in CKD patients with sepsis blocked FGF 23 (Dounousi et al., 2016)

Moreover, rats in group 3 -5 showed the more balance redox homeostasis than rats in group 2 with improved both Klotho mRNA expression and protein levels with lower score lesion from pathologic study in kidney tissue. It was implied that Vit E

and/or Vit C could protect renal handling of oxalate and the other promoters and inhibitors of CaOx crystal formation.

There is strong correlation between positive area analysis of kidney Klotho protein, reduced GSH, SOD and the lesion score in the kidney tissue from histology study. However, the soluble form in urine and plasma had no relation with other parameters. This could be results of many factors including dose and duration of the study, model of HLP induced hyperoxaluria and half-life and stability of the soluble Klotho protein.

## Limitation of the study

In the present study, we do not separate soluble form of KL1 and KL2 which has been identified earlier (Hu et al., 2015). Additionally, using Tiselius index for prediction of urinary supersaturation in hyperoxaluric rat model should be consider more than using the ratio between oxalate and citrate.

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# CHAPTER VI

# SUMMARY

In this present study, the administration of hydroxy-L-proline elevated the levels of oxalate in plasma and urine. Consequently, hyperoxaluria induced nephropathy by reduced glomerular filtration rate, reduced water and sodium reabsorption at the proximal tubule, enhanced CaOx crystal formation and increased oxidative stress. The administration of Vit E and/or Vit C could reverse these effects

In addition, kidney oxidative stress parameters were increased in hyperoxluric rats which could also be alleviated by administration of antioxidants. The results were agreed with renal histopathological data in which Vit E and/or Vit C improve renal tubular lesions caused by oxalate. The kidney protein Klotho expression was reduced while mRNA expression was enhanced in hyperoxaluric rats and these changes were improved after receiving antioxidants. Since there are strong correlations between oxidative stress parameters and either renal functions/structure or kidney Klotho protein expression, it is suggested that oxalate promoted oxidative stress which eventually reduced renal function and Klotho protein levels. By giving antioxidants, Vit E, Vit C or its combination, can alleviate renal impairment cause by hyperoxaluria.

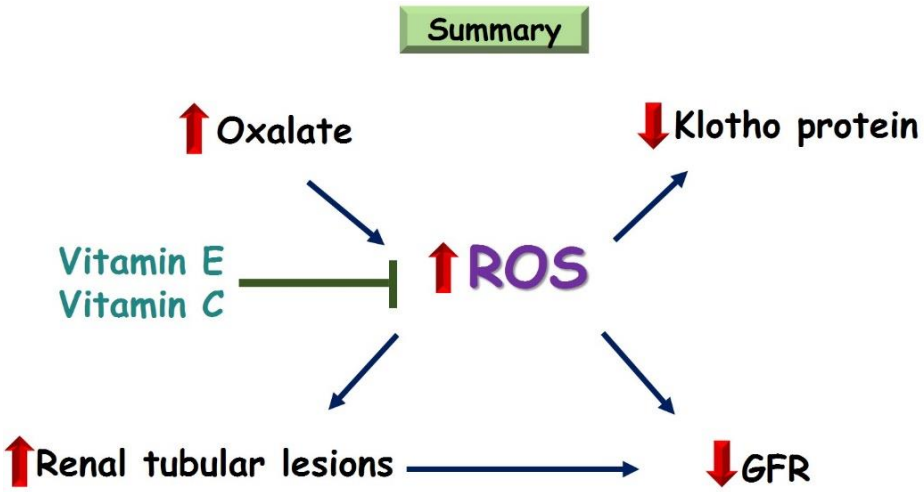


**Figure 6-1** Interplay between oxalate, ROS, renal function and Klotho protein. The supplement of Vit E and/or Vit C affects the relationships by suppress ROS formation.

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# ADDENDUM

Some data in part I of this study were published in The Journal of Veterinary Medical Science, issue 79, volume 5, page 896-903. Furthermore, some data in part II of this study were also submitted to The Journal of Veterinary Medical Science.

Some data in part II of this study was presented by oral and poster presentations in Chulalongkorn University Veterinary Conference 2017 at Queen Sirikit Convention Center, Bangkok, Thailand between 22-24 March 2017 entitle “Protective effects of vitamin E on oxidative stress, risk indices and Klotho protein in hyperoxaluric rats”.

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CONFERENCES / PRESENTATIONS:

Oral Presentation

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