ภาวะความเครียดออกซิเดชั่นและอะพอพโทซิสในไตของหนูแรทที่ได้รับซิสพลาตินและวิตามินซีในขนาดสูงมาก

นางสาวสุภา สิทธานุกูล

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RENAL OXIDATIVE STRESS AND APOPTOSIS IN RAT RECEIVING CISPLATIN AND MEGADOSE VITAMIN C

Miss Supa Sithanukul

A Thesis Submitted in Partial Fulfillment of the Requirements
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การทดลองในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของการใช้วิตามินซีในขนาดสูงมากร่วมกับซิสพลาตินต่อการทำงานของไตและภาวะความเครียดออกซิเดชั่นในหนูแรทพันธุ์สเปรย์ดอลเลย์ โดยแบ่งกลุ่มหนูที่ใช้ในงานวิจัยออกเป็น 4 กลุ่ม 1) กลุ่มควบคุม 2) กลุ่มที่ได้รับวิตามินซี 3) กลุ่มที่ได้รับซิสพลาติน และ 4) กลุ่มที่ได้รับซิสพลาตินร่วมกับวิตามินซี โดยทำการฉีดวิตามินซีในขนาด 1000 มิลลิกรัมต่อกิโลกรัมเข้าหลอดเลือดดำบริเวณหางในหนูกลุ่มที่ 2 และ 4 และทำการฉีดซิสพลาตินเข้าช่องท้องในขนาด 6 มิลลิกรัมต่อกิโลกรัมในหนูกลุ่มที่ 3 และ 4 ทำการเก็บเลือดและปัสสาวะสัตว์ทดลองทั้งหมดในวันก่อน (day 0) และหลังได้รับยาเป็นเวลา 6 วัน (day 6) นำไปวิเคราะห์หาค่าครีเอทินีน (P_{cr}) ค่ายูเรียไนโตรเจน (PUN) ค่าปริมาณของอิเล็กโตรไลท์ในพลาสมา เพื่อวิเคราะห์ค่าการทำงานของไตทั้งในส่วนของกลอเมอรูรัสและหลอดไตฝอย โดยนำมาคำนวณหาค่าการกรองผ่านกลอเมอรูลัสและค่าสัดส่วนการขับทิ้งของอิเล็กโทรไลท์ (FE Na^+, FE K^+, FE Cl^-) การวัดการเกิดความเครียดออกซิเดชั่นทำโดยตรวจหาปริมาณของมาลอนไดอัลดีไฮด์ (MDA) โปรตีนคาร์บอนิล (PC) และ total antioxidant status (TAS) ในพลาสมาและปัสสาวะ ส่วนในไตทำการวิเคราะห์โดยหาค่า MDA กลูตาไธโอน (GSH) และค่าการทำงานของเอมไซม์คะตาเลส (CAT) จากผลการทดลองพบว่าหนูกลุ่มที่ได้รับซิสพลาตินมีค่าการกรองผ่านกลอเมอรูลัสลดลงอย่างมีนัยสำคัญ ($P<0.05$) ในขณะที่มีการเพิ่มขึ้นของค่า P_{cr} ($P<0.05$) PUN ($P <0.05$) ค่าสัดส่วนการขับทิ้งของอิเล็คโตรไลท์ (FE) และอัตราการขับทิ้งโปรตีน ($P <0.05$) เมื่อเทียบกับกลุ่มควบคุม โดยค่าที่แสดงถึงความเสียหายของไตดังกล่าวสามารถลดลงได้ในหนูกลุ่มที่ได้รับซิสพลาตินร่วมกับวิตามินซี ในส่วนของการเกิดความเครียดออกซิเดชั่นนั้นพบว่า MDA ในพลาสมาเพิ่มขึ้นอย่างมีนัยสำคัญ ($P<0.05$) ในหนูกลุ่มที่ได้ซิสพลาตินเพียงอย่างเดียวเมื่อเทียบกับหนูในกลุ่มอื่น โดยทำให้เกิดการเพิ่มขึ้นของ MDA ร่วมกับการลดลงของ TAS ในปัสสาวะ เนื่องจากอัตราการกรองผ่านกลอเมอรูลัสมีความสัมพันธ์กับปริมาณ MDA ทั้งในพลาสมาและปัสสาวะ และสัมพันธ์กับ TAS ในปัสสาวะ ดังนั้นความเป็นพิษของซิสพลาตินต่อไตน่าจะเกิดขึ้นผ่านการเกิดความเครียดออกซิเดชั่นภายในไต การให้วิตามินซีทำให้การกรองผ่านกลอเมอรูลัสที่ลดลงจากฤทธิ์ของซิสพลาตินเพิ่มขึ้น ทั้งยังสามารถช่วยลดค่า MDA ในเลือดและปัสสาวะ และสามารถเพิ่มค่า TAS ในปัสสาวะที่มีค่าต่ำลงจากผลของยาซิสพลาติน อย่างไรก็ตามในการศึกษาค่าสัดส่วนของการแสดงออกของ mRNA ของ bcl-2 ต่อ bax ในเนื้อเยื่อไตพบว่าค่าสัดส่วนดังกล่าวลดลงหลังจากได้รับซิสพลาตินเป็นเวลา 6 วัน แสดงถึงการเกิดอะพอพโทซิสในไตที่เพิ่มสูงขึ้น และการใช้วิตามินซีไม่สามารถเปลี่ยนแปลงสัดส่วนการแสดงออกของยีนทั้งสองชนิดนี้ได้ จึงสามารถสรุปได้ว่าการได้รับซิสพลาตินจะทำให้เกิดความเสียหายของไตในส่วนของกลอเมอรูรัสและหลอดไตฝอยอย่างรุนแรง รวมถึงทำให้เกิดการเปลี่ยนแปลงของความเครียดออกซิเดชั่นและเพิ่มการเกิดอะพอพโทซิสภายในไต การให้วิตามินซีในขนาดที่สูงมากนี้สามารถช่วยลดผลจากความเป็นพิษของไตผ่านทางการลดการเกิดสภาวะเครียดออกซิเดชั่นได้แต่ไม่สามารถลดการเกิดอะพอพโทซิสจากผลของยา

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5375573331 : MAJOR ANIMAL PHYSIOLOGY
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SUPA SITHANUKUL: RENAL OXIDATIVE STRESS AND APOPTOSIS IN RAT RECEIVING CISPLATIN AND MEGADOSE VITAMIN C. ADVISOR: PROF. CHOLLADA BURANAKARL, DVM., Ph.D., CO-ADVISOR: CHUTAMAS BENJANIRUT, DVM., Ph.D., 61 pp.

The objectives of this study were to investigate the effects of megadose vitamin C in rats with cisplatin (CDDP) induced nephrotoxicity. Rats were divided into 4 groups as follows, group 1 (CONT), group 2 (vitamin C treated group; VIT C), group 3 (CDDP treated group; CDDP) and group 4 (CDDP + vitamin C treated groups; CDDP + VIT C). The vitamin C at the dose of 1000 mg/kg was given to the rats on day 1 in VIT C and CDDP + VIT C groups while CDDP was injected intraperitonealy at the dose of 6 mg/kg on day 1 in CDDP and CDDP + VIT C groups. The rats were subjected to renal function studies on one day before drug treatment (day 0) and 6 days after treatment (day 6). Blood and urine sampling were collected for measuring PUN and creatinine (P_{cr}) one day prior to renal clearance study in the metabolic cage. The GFR and fractional excretions of electrolytes were calculated. The oxidative stress markers, MDA, PC and TAS were measured in the plasma and urine while MDA, GSH and CAT were measured in the kidney. The results showed that at 6 day after CDDP administration, renal impairment as shown by significant reduction in GFR (P<0.05) with increased P_{cr} (P<0.05), PUN (P<0.05) and fractional excretion of electrolytes (Na^+, K^+ and Cl^-) and protein (P<0.05) were apparent as compared to control without CDDP. In CDDP-treated rats given vitamin C (CDDP + VIT C group), the GFR was improved significantly as compared with CDDP group. The P_{cr} and PUN levels also reduced although they were not significant different than CDDP group. By measuring the oxidative stress parameters, the plasma MDA increased in CDDP group significantly (P<0.05) when compared with other groups. Urinary MDA/Cr also increased along with decreased TAS. The GFR value was correlated with PMDA, urinary MDA/Cr and urinary TAS. Giving vitamin C in CDDP-treated rats (CDDP + VIT C group) showed improved GFR and lower PMDA, urinary MDA/Cr with increased urinary TAS. However, the PCR products of antiapoptosis/proapoptosis (bcl-2/bax) which was reduced 6 days after CDDP was unchanged. These results suggest that CDDP cause severe glomerular and tubular damage, renal apotosis with enhanced renal oxidative stress. CDDP induced renal impairment may be mediated via renal oxidative damage. Giving megadose vitamin c in CDDP treated rats could alleviate the effects of CDDP on renal functions but not renal apoptosis via reduction in renal oxidative stress.

Department:	Veterinary Physiology	Student's Signature	....
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LIST OF ABBREVIATIONS

A II	angiotensin II
AA%	antioxidant activity percent
ANOVA	analysis of variance
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
Bax	pro-apoptotic regulatory gene
Bcl-2	anti-apoptotic regulatory gene
BHT	butylatedhydroxytoluene
$[Ca^{2+}]I$	intracellular calcium level
CAT	catalase enzyme and activity
CDDP	cisplatin
Cdk2	cyclin-dependent kinase 2
cDNA	complementary DNA
Cl^-	choride
C_{H2O}	free water clearance
C_{osm}	osmolar clearance
DFO	deferoxamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine
DPPH	a,a-Diphenyl-b-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid

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EGTA	ethylene glycol tetraacetic acid
FE_e	fractional excretion of electrolyte
FE Cl^-	fractional excretion of chloride
FE K^+	fractional excretion of potassium
FE Na^+	fractional excretion of sodium
g	gram
GdmCl	guanidine hydrochloride
GFR	glomerular filtration rate
GSH	reduced glutathione
Hct	hematocrit
HCl	hydrogen chloride, hydrochloric acid
H_2O_2	hydrogen peroxide
In vivo	latin for "within the living"
In vitro	latin for "in glass"
i.p.	intraperitoneal
i.v.	intravascular
K^+	potassium
KCl	potassium chloride
Kg	kilogram
L	litter
O_2	oxygen
$O_2^{\bullet -}$	superoxide anion
$OH^\bullet$	hydroxyl radical
MDA	malondialdehyde

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mEq	milliequivalents
mg	milligram
mgCr	milligram of creatinine
min	minute
ml	milliliter
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
Na^+	sodium
NAD(P)H	nicotinamide adenine dinucleotide phosphate-oxidase
nmol	nanomoles
PBS	phosphate-buffered saline
PC	protein carbonyl
P_{cr}	plasma creatinine
PCR	polymerase chain reaction
PUN	plasma urea nitrogen
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TAS	total antioxidant status
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reacting substances
TCA	trichloroacetic acid
Tiron	1,2-dihydroxy-3,5-benzene- disulphonate

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UPC	urinary protein to creatinine ratio
μg	microgram
μl	microliter
μmol	micromoles

CHAPTER I

INTRODUCTION AND AIMS

Cisplatin (cis-diamminedichloroplatinum II, CDDP) has been used for chemotherapeutic treatment protocols for many types of cancer. However, CDDP and related platinum-based anticancer drugs caused nephrotoxicity, ototoxicity, neurotoxicity and anemia (Pabla and Dong, 2008; Miller et al., 2010; Amptoulach and Tsavaris, 2011; Mukherjea and Rybak, 2011; Sanchez-Gonzalez et al., 2011). Although second-generation drugs were developed to reduce their toxicity and improve clinical outcomes of platinum compounds, CDDP remains as a regular component for the treatment of some specific cancers (Homma et al., 2011; Tsan et al., 2012). The CDDP causes cytotoxic effect by modifying DNA structure involving in DNA-damage recognition and DNA repair leading to apoptosis (Siddik, 2003). However, apoptosis was reported in renal tissue after using CDDP. CDDP-induced renal epithelial cells apoptosis involves multiple pathways including both extrinsic pathway, the intrinsic mitochondrial pathway and the endoplasmic reticulum stress pathway (Miller et al., 2010). It has been demonstrated that oxidative stress may play a central role in renal toxicity of platinum-based anticancer drugs (Santos et al., 2007; Santos et al., 2008; Chirino and Pedraza-Chaverri, 2009; Lin et al., 2010; Rubera et al., 2013). Using antioxidants in combination with chemotherapy could diminish the risk of CDDP-induced nephropathy by lower oxidative damage (Antunes et al., 2000; Tsuruya et al., 2003; Cetin et al., 2006; Ajith et al., 2007; Santos et al., 2008; Tarladacalisir et al., 2008; Ajith et al., 2009).

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Vitamin C (ascorbic acid) is water-soluble antioxidant in human plasma and mammalian cells (Padayatty et al., 2003) and a coenzyme in all metabolism (Kojo, 2004; Duarte and Lunec, 2005). Interestingly, high-dose vitamin C is considered to be one of an alternative therapies for cancer since its selectively toxic to tumor cell *in vitro* and *in vivo* (Riordan et al., 1995). The level of ascorbate radical and hydrogen peroxide were elevated in extracellular fluid *in vivo* (Chen et al., 2007). Moreover, vitamin C can induce the generation of oxalate (Pena de la Vega et al., 2004; Cossey et al., 2013) and associated with oxalate nephropathy (Lamarche et al., 2011; Gurm et al., 2012; Poulin et al., 2014). Vitamin C involved in the oxidative damage in renal epithelial cell (LLC-PK_1 cells) (Scheid et al., 1996; Thamilselvan et al., 2003).

In practice, a combination of ascorbic acid and carboplatin was also used for treatment of ovarian cancer (Drisko et al., 2003). Previous studies showed that vitamin C may increase antineoplastic activity of CDDP in human breast carcinoma cells in vitro (Kurbacher et al., 1996) and increase the apoptosis via up-regulation p53 in human colon cancer cells *in vitro* (An et al., 2011). However, the adverse effects of CDDP in combination with megadose vitamin C on nephrotoxicity have not yet been investigated in relation to changes in oxidative stress and apoptosis of renal tissue.

The objectives of the present study were to investigate; firstly, to study the effects of using either CDDP or megadose vitamin C alone on renal functions, renal oxidative stress and renal apoptosis in rat; secondly, to study the effects of CDDP in combination with megadose vitamin C on renal functions, renal oxidative stress and renal apoptosis in rat.

Hypotheses

1. A combination of CDDP and megadose vitamin C causes a further impairment of renal function compared with single drugs administration.
2. A drugs combination increases more renal oxidative stress.
3. A drugs combination alters renal Bcl-2 to Bax mRNA ratio.

CHAPTER II
LITERATURE REVIEWS

Cisplatin pharmacology and action

Cisplatin (CDDP) is a platinum base anticancer drug used to treat various types of solid tumors. The structure was shown in figure 1.

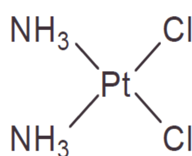

Figure 1. Structure of cisplatin

Platinum compounds enters the cell by passive diffusion (Gately and Howell, 1993) and through the copper transporter CTR1. The two copper efflux transporters ATP7A and ATP7B was responsible for the efflux of platinum drugs (Safaei and Howell, 2005; Kelland, 2007). Once the drug entering into the cytoplasm, water molecule reacts with the drug resulting in a chemically reactive aqua species. This mechanism is called "aquation" and is the rate limiting step in the reaction between platinum compounds and DNA. The dose of platinum base anticancer drug used was depends upon the rate of aquation (Knox et al., 1986).

High concentration of platinum is found in the kidney, liver and skin 6 days after the administration of CDDP and persists for at least 10 days in the tissues of mice (Siddik et al., 1988). CDDP had half life of 13 minutes in the first phase ($t_{1/2}$ alpha), followed by an elimination half life of 43 minutes in the second phase ($t_{1/2}$ beta), and a terminal half life of 5.4 days in the third phase ($t_{1/2}$ gamma) in human patients (Go and Adjei, 1999). The aquated platinum complex can then form

crosslink with DNA at the N7 position of purine base by the covalent binding. The formation of platinum-DNA adducts caused DNA lesion consisting of monoadducts, intrastrand crosslinks and interstrand crosslinks (Rabik and Dolan, 2007). The DNA damage results in replication arrest, transcription inhibition, cell-cycle arrest, DNA repair and apoptosis/cell death. The active aqua species can also react with various macromolecules, particularly high sulphur-containing molecules including tripeptide glutathione and metallothioneins (Wang and Lippard, 2005; Kelland, 2007)

Clinical usage of cisplatin

CDDP has been widely used for more than 30 years as a part of treatment regimen such as head and neck, lung, testis, ovary and breast cancers (Pujol et al., 2000; Argiris et al., 2005; Azzoli et al., 2009; Helm and States, 2009; Riese and Vaughn, 2009; Koshy et al., 2010; Miller et al., 2010). In dogs, CDDP has been used to treat osteosarcoma, squamous cell carcinoma, transitional cell carcinoma, nasosinus carcinoma and melanoma (Barabas et al., 2008). However, CDDP is not used in cat due to severe pulmonary toxicity (Knapp et al., 1987). In Thailand, CDDP is used as a part of therapeutic regimen for several types of cancer (Thongprasert et al., 2005; Punushapai et al., 2010; Thongprasert et al., 2011).

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Cisplatin nephrotoxicity

The mechanisms responsible for the pathogenesis of platinum anticancer drugs induced renal injury have not yet been fully investigated. CDDP nephrotoxicity is often seen after 10 days of the drug administration, as shown by decreased

glomerular filtration rate (GFR), increased serum creatinine concentration, and reduction of serum magnesium and potassium concentration (Pabla and Dong, 2008). Another study showed that the serum concentration of creatinine, blood urea nitrogen (BUN) and tubular damage score were significantly increased on day 5 after CDDP administration in rat at the CDDP dose of 6 mg/kg (Zhou et al., 2006).

Cisplatin-induced oxidative stress

Oxidative stress is the situation with high levels of reactive oxygen species (ROS), such as $O_2^{\bullet-}$ (superoxide), $OH^{\bullet}$ (hydroxyl radical) and H_2O_2 (hydrogen peroxide). This is originated by an overproduction of free radicals or by an imbalance between production of ROS and antioxidant defenses (Djordjevic, 2004). The formation of superoxide anion radical ($O_2^{\bullet-}$) (figure 2) can be mediated by NADPH oxidases and xanthine oxidase or non-enzymatically by the mitochondrial electron transport chain. The $O_2^{\bullet-}$ is dismutated to hydrogen peroxide (H_2O_2) by the superoxide dismutase (SOD) and changes into hydroxyl radical ($OH^{\bullet}$) via Fenton reaction. However, H_2O_2 can be neutralized to H_2O and O_2 by GSH (reduced glutathione) and catalase (Valko et al., 2007). Some of the commonly used oxidative stress markers are listed as following (Ogino and Wang, 2007; Chirino and Pedraza-Chaverri, 2009).

1. Malondialdehyde (MDA) as an indicator for lipid peroxidation.
2. Protein carbonyl groups are used as biomarkers of protein oxidation.
3. Antioxidant depletion. (Namely, catalase, glutathione reductase, etc.)

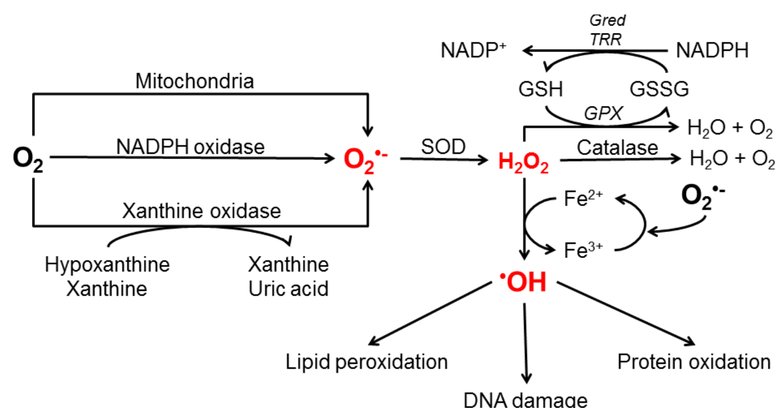

Figure 2. The mechanism of ROS generation.

Ajith et al. (2009) evaluated the protective effect of vitamin C and E on CDDP-induced acute renal failure in mice. Administration of single and multiple doses of these two vitamins showed a protective effect against CDDP-induced nephrotoxicity.

1.) Role of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ on cisplatin-induced oxidative stress

There are number of studies supporting the role of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ in cytotoxicity and cellular alterations caused by CDDP exposition. The mechanism of CDDP-induced cell death in primary cultures of mouse proximal tubular cells showed that ROS plays a role in CDDP-induced apoptosis by decreased apoptosis due to the addition of various antioxidants and scavengers of ROS. The $O_2^{\bullet-}$ scavengers (SOD and 1,2-dihydroxy-3,5-benzene- disulphonate (tiron)) and $OH^{\bullet}$ scavengers (deferoxamine (DFO), dimethyl sulfoxide (DMSO) and probucol) can reduce CDDP-induced apoptosis in mouse proximal tubular cells (Lieberthal et al., 1996). Overexpression of mitochondria-localized manganese superoxide dismutase (MnSOD) alone with decreased renal injury supported the role of $O_2^{\bullet-}$ for cellular damage (Davis et al., 2001).

2.) Role of iron on cisplatin-induced oxidative stress

Iron has a major role in lipid peroxidation. It promotes the formation of perferryl ion leading to biomolecule damage and removes of hydrogen atoms from polyunsaturated fatty acids in the lipid bilayers of organelle membranes. Thus, iron directly initiates and propagates lipid peroxidation (Shah et al., 2011). Baliga and coworkers (1998) investigated the effect of iron chelators on the catalytic iron content in an *in vivo* model of rats and an *in vitro* model of LLC-PK1 cells (renal tubular epithelial cells). The results showed that exposure of CDDP to LLC-PK1 cells could release of iron into the medium. Iron chelators, such as DFO and 1,10-phenanthroline, could also reduce CDDP-induced cytotoxicity. In vivo study showed that DFO has a protective effect against CDDP-induced acute kidney injury in their rat model. Furthermore, DMSO, also have a protective effect on CDDP-induced toxicity both *in vitro* and *in vivo*, which support the role of iron on CDDP-induced nephrotoxicity via $OH^{\bullet}$ (Baliga et al., 1998).

3.) Role of cisplatin-induced oxidative stress on mitochondria function

From the information of cellular target of CDDP above, mitochondrial dysfunction is considered as a key role in CDDP-induced nephrotoxicity. The CDDP inhibits complexes I to IV of the mitochondrial respiratory chain, and elevates the formation of superoxide anion. The $O_2^{\bullet-}$ may originate hydroxyl radical via Fenton reaction leading to oxidative stress (Kruidering et al., 1997). The role of mitochondrial ROS generation on CDDP-induced oxidative stress is evidenced by the ability of mitochondrial-targeted antioxidants, namely, MitoQ and Mito-CP which prevent CDDP-induced nephrotoxicity in a dose-dependent manner in mouse model (Mukhopadhyay et al., 2012).

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4.) Role of calcium on cisplatin-induced oxidative stress

Kawai et al. (2006) reported that CDDP at 500 µM significantly increased the intracellular calcium level ([Ca^{2+}]i) prior ROS production in renal epithelial cells (LLC-PK1). In addition, BAPTA-AM (1,2-bis(O-aminophenoxy) ethane-N,N,N',N'-tetra acetic acid tetra (acetoxymethyl) ester), an intracellular calcium-chelating compound, inhibited both ROS production, cell injury and also reduced [Ca^{2+}]i level. However, an extracellular calcium chelator, including ethylene glycol tetraacetic acid (EGTA) and a calcium channel blocker, nicardipine, could not inhibit an increase of [Ca^{2+}]i level. The results suggested the calcium from the intracellular storage was more important in the generation of ROS and cell injury.

Cisplatin-induced apoptosis

The CDDP induces renal epithelial cell death through several apoptotic pathways, including the extrinsic pathways, and the intrinsic mitochondrial pathway and the endoplasmic reticulum stress pathway (Miller et al., 2010). In the intrinsic pathway, the B cell lymphoma 2 (Bcl-2) family controls the pathway by acting as a checkpoint upstream of caspase activation and mitochondrial dysfunction (Chao and Korsmeyer, 1998).

Bcl-2 is an anti-apoptotic members of the Bcl-2 family (Gross et al., 1999). The overexpression of Bcl-2 could suppress CDDP-induced apoptosis in rat neuroblastoma cell line (Park et al., 2001). Another study demonstrated the induction of Bcl-2 by uranyl acetate with attenuated renal cell apoptosis (Zhou et al., 1999). Bax (Bcl-2-associated X protein) is a pro-apoptotic member of the Bcl-2

family (Gavathiotis et al., 2008). The expression of Bax induces alteration in mitochondrial membrane potential, production of ROS and releasing of mitochondrial cytochrome c. These situations activate a downstream caspase program leading to cell death (Xiang et al., 1996; Pastorino et al., 1998; Gross et al., 1999). Wei et al. (2007) studied the pathological role of Bax in CDDP-induced nephrotoxicity using Bax knockout mice, which showed that the number of apoptotic cells decreased in the Bax-deficient mice compared to the wild-type mice. In addition, the release of mitochondrial cytochrome c was suppressed in primary cultures of proximal tubular cells.

It has been proposed that ratio of Bax to Bcl-2 regulates the sensitivity of cells to apoptotic stimuli and is a checkpoint in the cell death pathway (Gross et al., 1999). Oltvai et al. (1993) reported that the overexpression of Bax promoted apoptotic cell death and suppressed Bcl-2 anti-apoptotic activity. In contrast, the overexpression of Bcl-2 repressed apoptotic death by the heterodimerization of Bcl-2 and Bax (Korsmeyer, 1999). Study in rat kidney by Sheikh-Hamad et al. (2004) showed that administration of CDDP (5 mg/kg) did not alter the Bcl-2 mRNA expression within 5 days while mRNA Bax were significantly increased, resulting in decreasing of Bcl-2/Bax mRNA ratio.

. Cell cycle regulators also have an essential role in tubular cell damage. Price and coworkers (2006) demonstrated that the cytotoxic effect of CDDP depended on the activation of cyclin-dependent kinase 2 (cdk2). However, the drug promoted the expression of p21, a cyclin-dependent kinase inhibitor which plays a protective role against CDDP-induced toxicity by direct inhibition of cdk2 (Megyesi et al., 1998; Price et al., 2006).

Vitamin C pharmacology

Vitamin C (ascorbic acid, ascorbate) is a potent water-soluble antioxidant which it structure was shown in figure 3.

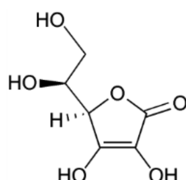

Figure 3. Structure of vitamin C

Vitamin C is absorbed in the intestine using Na^{+}-dependent active transporter (Rivers, 1989). By oral route, plasma concentration of vitamin C does not exceed 100 μM due to limit absorption, ascorbate bioavailability and enhanced urine excretion (Levine et al., 1996). On the other hand, higher plasma concentration can be reached by intravenous administration. Since the higher ascorbate intake results in more rapid ascorbate excretion, therefore the plasma half-life of ascorbate is dose dependent (Duconge et al., 2008). Ascorbate and its metabolites are eliminated by the kidney (Rivers, 1989).

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Ascorbate is easily oxidized into dehydroascorbate form and can be regenerated back using NADPH-dependent ascorbate recycling in mammalian cell (Duconge et al., 2008). The initial process is the hydrolysis of dehydroascorbate to 2,3-diketo-l-gulonate follows by the degradation of 2,3-diketo-l-gulonate into oxalate, CO_2 and l-erythrulose (Linster and Van Schaftingen, 2007). However, in pathologic conditions dehydroascorbate was accumulated. Since dehydroascorbate is highly unstable at physiological pH, this compound will rapidly decomposed unless it is recycled to ascorbate (Duconge et al., 2008).

The cytotoxic effect of vitamin C

The cytotoxic effects of ascorbate seem to be mediated by H_2O_2 generation. The formation of H_2O_2 from ascorbate can be enhanced by divalent cation such as iron via fenton reaction (Chen et al., 2007). Furthermore, the level of H_2O_2 generation was depended upon the ascorbate concentration and serum antioxidants. Therefore, ascorbate can act either a pro-oxidant or an antioxidant agent depending on the condition of the cells (Duconge et al., 2008). Previous study demonstrated that ascorbate radical and hydrogen peroxide were after the administration of a single pharmacologic dose of ascorbate (4 g/kg of body weight) intravenously in cancer cell (Chen et al., 2008). On the contrary, H_2O_2 will be changed into H_2O and O_2 in blood which has high level of antioxidant enzyme (Chen et al., 2007). It was known that tumor cells are more sensitive to hydrogen peroxide than normal cell since they lack of catalase enzyme (CAT) (Gonzalez et al., 2005).

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Vitamin C induced nephropathy

Oxalate is a relatively useless end product of ascorbic acid metabolism in human (Hellman and Burns, 1958). Oxalate nephropathy can occur as a result of the intratubular crystallization of calcium oxalate.

Hyperoxaluria could induce acute tubular necrosis and hence acute kidney injury. Primary hyperoxaluria is characterized by the overproduction of oxalic acid because of excessive intake or increased intestinal absorption of oxalate causing secondary hyperoxaluria (Lamarche et al., 2011). The exposition of oxalate to renal cell induces over production of ROS followed by renal injury and inflammation (Khan, 2005). Reports of acute oxalate nephropathy after intravenous vitamin c were

found (Wong et al., 1994; Padayatty et al., 2010). The intravenous ascorbic acid produces less oxalic acid than the high-dose oral ascorbic acid administration. Ascorbate could remain in the alkaline medium of the intestine for a long time, before absorbed into the circulation (Robitaille et al., 2009).

Previous study described the present of oxidative damage after vitamin C exposure (Verrax and Calderon, 2008). Duarte and Jones (2007) demonstrated that ascorbic acid altered cellular iron homeostasis and promoted intracellular Fenton reaction under oxidative stress leading to further increased oxidative damaged in normal human diploid fibroblasts (HDFs).

The combination of platinum-base anticancer drug and vitamin C

The clinical usage of combination of vitamin c and cisplatin-derivative, carboplatin for the treatment of advance ovarian cancer was reported (Drisko et al., 2003). Many studies suggest that vitamin C might be benefit of CDDP in cancer therapy. The study from An et al. (2011) showed that vitamin C (100 µg/ml) increased the sensitivity of CDDP in human colon cancer cells (HCT116) and promoted apoptosis via the upreguration of p53. Addition of ascorbic acid improved antineoplastic effect of CDDP on human breast carcinoma cells *in vitro* (Kurbacher et al., 1996). Unfortunately, the adverse effects of drugs combination on renal impairment and apoptosis in relation to oxidative stress have not yet been fully investigated.

CHAPTER III
MATERIALS AND METHODS

Experimental Animals

The experiment was performed in accordance with the institutional guidelines and conformed to CU-IACUC, Faculty of Veterinary Science, Chulalongkorn University. The male Sprague Dawley rats, weighing between 250-300 g were used. The animals were obtained from the National Laboratory Animal Center, Mahidol University (NLAC-MU) and were housed under standard condition of light and dark cycle (L:D = 12:12) with free access to food and water. The rats were acclimatized to the laboratory condition for seven day prior to the experimental use and randomly divided into four groups as follows:

Group 1 – Control (CONT) group (n=9), rats received an intraperitoneal injection of isotonic saline (0.6 ml/100g body weight) just before an intravenous injection of isotonic saline (0.4 ml/100g body weight).

Group 2 – Cisplatin (CDDP) group (n=9), rats received an intraperitoneal injection of cisplatin (6 mg/kg body weight) followed by an intravenous injection of isotonic saline (0.4 ml/100g body weight).

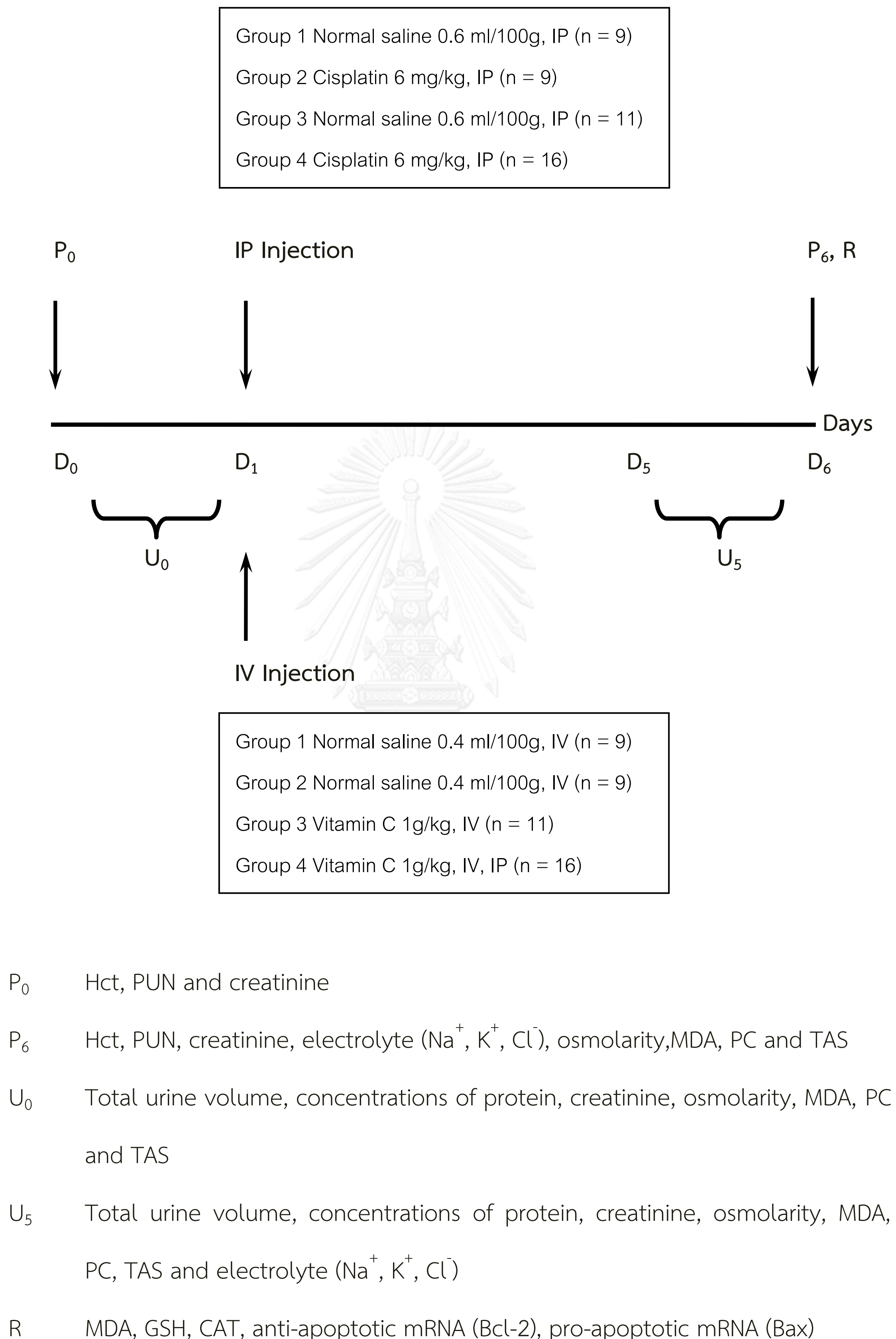
Group 3 – Megadose vitamin C (VIT C) group (n=11), rats received an intravenous injection of vitamin c (1000 mg/kg body weight) after an intraperitoneal injection of isotonic saline (0.6 ml/100g body weight).

Group 4 – Cisplatin + Megadose vitamin C (CDDP + VIT C) group (n=16), rats received an intraperitoneal injection of cisplatin (6 mg/kg body weight) just before the intravenous injection of vitamin c (1000 mg/kg body weight).

Experimental protocol

During the experimental period, body weight and food intake were recorded daily. One day before drug administration (D_0) 0.5 ml of blood (P_0) was collected from each rat by clipping the tip of the tail. The blood was used for the measurement of hematocrit (Hct), plasma urea nitrogen (PUN) and creatinine (P_{cr}). Each rat was housed in a metabolic cage in order to collect urine sample (U_0) on the next day to measure total urine volume, concentrations of protein, creatinine, osmolarity, malondialdehyde (MDA), protein carbonyl (PC) and total antioxidant status (TAS). After housing the rat for 24 hours (D_1), each rat was restrained and anesthetized with pentobarbital sodium (60 mg/kg b.w., i.p.).The intraperitoneal administration was performed followed by intravenous administration. The rat was placed in the regular cage with food and water supplied *ad libitum.*

Four days after drug administration (D_5), the animals were housed in metabolic cage for 24 hours for urine collection again (U_5) to measure total urine volume, concentrations of protein, creatinine, osmolarity, MDA, protein carbonyl, TAS and electrolyte (Na^+, K^+, Cl^-). On day 6 (D_6) after the collection, each rat was anesthetized with pentobarbital sodium (60 mg/kg b.w., i.p.). The 5 ml of blood (P_6) was collected by cardiac puncture for the measurement of Hct, PUN, creatinine, electrolyte (Na^+, K^+, Cl^-), osmolarity, MDA, PC and TAS. Both kidneys were excised and kept on -70° c for analyses of oxidative stress and apoptotic markers. Systolic blood pressure measurement was performed using the tail-cuff method before anesthetized at D_1 and D_6.



P_0 Hct, PUN and creatinine

P_6 Hct, PUN, creatinine, electrolyte (Na^+, K^+, Cl^-), osmolarity,MDA, PC and TAS

U_0 Total urine volume, concentrations of protein, creatinine, osmolarity, MDA, PC and TAS

U_5 Total urine volume, concentrations of protein, creatinine, osmolarity, MDA, PC, TAS and electrolyte (Na^+, K^+, Cl^-)

R MDA, GSH, CAT, anti-apoptotic mRNA (Bcl-2), pro-apoptotic mRNA (Bax)

Analytical procedures for determinations of blood, urine and kidney samples

Plasma and urinary Na^{+} and K^{+} concentrations were measured using flame photometer (Flame photometer 410C, Ciba Corning Inc., USA). The Cl^{-} concentration in both plasma and urine were determined by chloridometer (Chloride analyzer 925, Ciba Corning Inc., USA). Urine and plasma osmolarity were determined using osmometer (Osmometer 3D3; Advance Instruments Inc.,Norwood, MA, USA). Systolic blood pressure was measured by the tail-cuff method. Plasma concentration of urea nitrogen, plasma and urinary creatinine concentrations were measured by colorimetric method using the automate analyzer. A urinary protein concentration was measured by precipitating with sulfosalicylic acid. Kidney protein concentration was measured by Lowry et al. (1951) for MDA and Bradford et al. (1976) for CAT measurement. Plasma and urinary TAS were determined by a modification method as described by Miller et al. (1993). Plasma and urinary protein carbonyl were measured by spectrophotometric dinitrophenylhydrazine (DNPH) assay following a modification method of Levine et al. (1990). MDA in plasma, urine and kidney were determined by a modification method of Marshall et al. (1985). Renal GSH content was determined following a modification method of Beutler et al. (1963). Renal CAT activity was measured by a modification method of Aebi (1983). Renal Bcl-2 and Bax mRNA expression were measured by real-time PCR.

Procedure for measurements of TAS in plasma and urine

A modification method of Miller et al. (1993) was performed to measure the TAS in plasma and urine samples. These assays are based on the interaction

between a stable free radical a,a-Diphenyl-b-picrylhydrazyl (DPPH) and antioxidant enzymes in the samples. Twenty microliters of plasma or urine samples was added to the mixture of 400 µl sodium phosphate buffer and 400 µl DPPH, then mixed carefully and incubated at the room temperature for 20 min. The absorbance was measured at 520 nm. The radical scavenging activity (% inhibition) was showed as percentage of DPPH radical elimination which is calculated by the following formula; % inhibition= $[(OD_{blank} - OD_{test})/ OD_{blank}]\times 100$.

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Procedure for measurements of protein carbonyl in plasma and urine

The spectrophotometric dinitrophenylhydrazine (DNPH) assay was performed to measure urinary protein carbonyl as described by Levine et al. (1990). The samples were diluted 1:20 with phosphate-buffered saline (PBS) and centrifuged 10,000 rpm for 15 min. The supernatant (250 µl) was added in a solution of 250µl DNPH in 2 N HCl. Samples were kept in the dark at room temperature for 45 min with vortexing every 5 min. The mixtures were precipitated with cold trichloroacetic acid (TCA, 20%) and then refrigerated for 20 min followed by centrifuged at 10,000 rpm for 5 min. The supernatants were removed, the protein pellets were washed three times with ethanol:ethyl acetate (1:1 mixture), then 1 ml guanidine hydrochloride (GdmCl) was added to the samples. Lastly, the mixtures were sonicated to dissolve the sediment and the absorbance was read at 370 nm. Plasma and urinary protein carbonyl were expressed as nmol per milligram of protein calculated using the extinction coefficient of DNPH ($22,000 M^{-1}/cm$) at 370 nm. The

carbonyl concentration was calculated using the formula as described below. The result was expressed as nmol/ml

The carbonyl concentration (in moles L^{-1}) = [(Abs at 370nM)/22,000] $\times 10^{6}$

Procedures for measurements of MDA in kidney, plasma and urine

Malondialdehyde (MDA) was assayed in the form of thiobarbituric acid reacting substances (TBARS). TBARS was determined using a modification method of Ohkawa et al. (1979). The renal cortex was homogenized in 1.15% potassium chloride plus 0.003 M EDTA (1:10 w/v). The homogenate was centrifuged at 3,000 g for 30 minutes. Four hundred microliters of supernatant was added to a reaction mixture containing 8.1% sodium dodecyl sulfate (SDS) 200 µl, 50mM butylatedhydroxytoluene (BHT) in absoluted ethanol 50 µl, 20% acetic acid 750 µl, 0.5% 2-thiobarbituric acid (TBA) 750 µl and water 50 µl. The mixture was heated in a boiling water bath for 60 minutes and then cooled on ice for 5 minutes. Two milliliter of n-butanol:pyridine (15:1) was added to the mixture and mixed vigorously for 1 minute. After centrifugation at 5,000 rpm for 10 minutes, the absorbance of the supernatant was measured at 532 nm by spectrophotometer. MDA value was expressed as nmol of TBARS per milligram protein which is determined by Lowry method.

To determine plasma MDA concentration, 500 µl of plasma was employed with MDA assay described above. Urine MDA was obtained using 500 µl samples of urine.

Procedure for measurement of kidney GSH

Reduced glutathione (GSH) was determined using a modification method of Beutler et al. (1963). Renal cortex tissue (0.5 g) was homogenized in 4.5 ml of 100 mM KCL with 0.003 M EDTA. The homogenate was centrifuged at 600 g for 10 min. The 1 ml of supernatant was added to 1.5 ml metaphosphoric acid and removed particulate debris by centrifugation at 3,000 g for 10 min. Five hundred microliters of supernatant was added to 2.0 ml of 0.2 M phosphate buffer and 0.25 ml 0.04% 5,5'dithio-bis 2-nitrobenzoic acid (DTNB). Absorbance was read at 412 nm. The result was expressed as unit per milligram protein.

Procedure for measurement of kidney catalase activity

Kidney catalase activity (CAT) was determined using a modification method of Aebi (1983) and was expressed as sec^{-1} per milligram homogenate protein. Slice of renal cortex tissue (0.5g) was homogenized with 1% Triton X-100. One hundred microliters of supernatant was 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 read at 240 nm every 30 sec for 1-2 min using UV-VIS spectrophotometer. Catalase activity was expressed as sec^{-1} per milligram protein which was determined by Bradford protein assay.

Procedure for measurements of Bcl-2 and BAX in the kidney

After anesthetized by pentobarbital sodium (60mg/kg), the rat kidneys was quickly removed and stored at -70°C for RNA extraction. Total RNA was extracted from 20 mg frozen kidneys using Total RNA Mini Kit (Tissue) (Geneaid Biotech Ltd.).

Spectrophotometer was used for quantitating the amount of RNA. Readings was taken at wavelengths of 260 nm and 280 nm. Total kidney RNA was reverse transcribed using Random primers and iScript Select cDNA synthesis Kit (Bio-rad). The first-strand synthesis cDNA obtained was synthesized from 1 µg of total RNA as the manufacturer's instruction (Bio-rad laboratories). Two µl of the first-strand synthesis cDNA was used as templates and amplified by real time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Forter City, CA, USA) with primers for rat Bcl-2, BAX, and GAPDH genes. These reactions were performed in 0.2 ml 96 wells plate in the thermal cycler sample block of ABI 7500 Real-time PCR machine (Applied Biosystems, Forter City, CA, USA). The initial steps, for activation Real-time-PCR Master Mix E4 (GeneOn); at 95°C for 10 minutes and the typical two-temperature cycle for a PCR was run which consists of a denature step at 95°C for 15 seconds, followed by an anneal-extension step at 60°C for 1 minute. Each reaction was amplified in triplicate and a control without cDNA was performed in parallel with each assay. The relative changes of the interested genes expression and the ratio were calculated base on the comparative C_T method ($2^{-\Delta\Delta CT}$ method). The housekeeping gene (GAPDH) was used as an internal control. The sequences of specific primers are shown in table 1.

Table 1. Sequences of primers used in RT-PCR.

Genes	Primer sequence 5'- 3'	Accession number	Size of product (bp)	Reference
Rat Bcl-2	F - GGGATGCCTTTGTGGAACTATATG	NM016993	62	Cheng et al. (2009)
	R - CAGCCAGGAGAAATCAAACAGA			
Rat Bax	F - ATGGAGCTGCAGAGGATGATT	NM017059	97	Ding et al. (2011)
	R - TGAAGTTGCCATCAGCAAACA			
Rat GAPDH	F - TCCCTCAAGATTGTCAGCAA	NM017008	309	Gong et al. (2008)
	R - AGATCCACAACGGATACATT			

Two and four percentage agarose gel electrophoresis were performed to confirm the product size of each primer.

Calculation

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

Urinary excretion of substance = $U_x V$

Fractional excretion of electrolyte (FE_e) = $\frac{U_eV / P_e \times 100}{GFR}$

C_{osm} = $U_{osm} V / P_{osm}$

C_{H2O} = $V - C_{osm}$

Statistical Analysis

All numerical data were expressed as mean ± S.E. One-way analysis of variance (One-way ANOVA) was used to compare the data between groups and followed by post-hoc analysis with Student Newman-Keuls method. To compare

data in the same group at different time point, paired t-test or Wilcoxan signed-rank test was used. The relationships between parameters were determined using linear regression and polynomial regression equations. The P value of less than 0.05 was considered to be statistically significant.

CHAPTER IV

RESULTS

Body weight, food intakes and water intakes

The body weights before and after treatments in each group of rats were shown in figure 4. Mean weights were similar among groups before treatments at day -1 and 0. After 5 days of treatments (day 6), the body weights of both CDDP and CDDP + VIT C group were significantly lower than before treatments ($p<0.001$). When comparing the body weight among groups, body weights in CDDP group was lower significantly compared with other groups at day 5 and 6 after CDDP treatments ($P<0.05$).

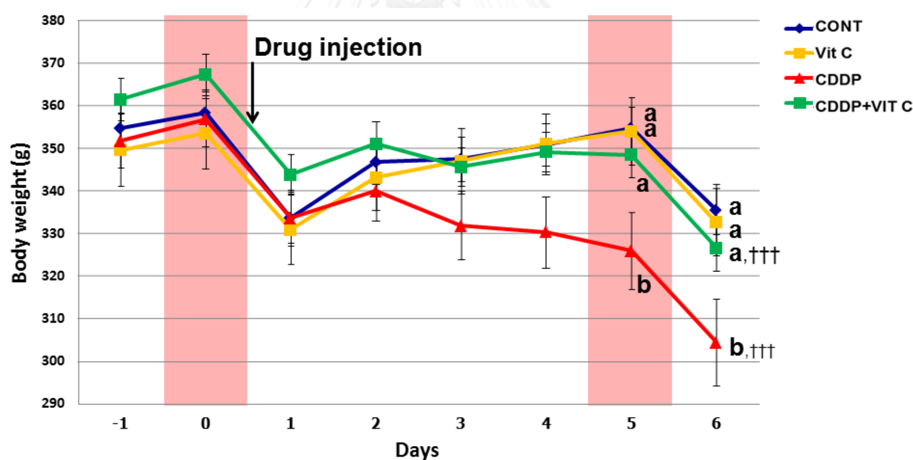

Figure 4. Daily mean values for body weight in four groups of rats throughout the experimental period.

Different superscripts means differ significantly ($p<0.05$) among groups at the same day using one-way ANOVA followed by Student Newman-Keuls method.

††† = $P<0.001$ significant difference from the values at the day of drug injection (day 1) using Wilcoxon signed rank test.

The shade area showed rats in metabolic cage.

Food intakes before and after treatments in each group of rats were presented in figure 5. Mean food intakes were similar among groups before treatments. After drug administration, food intake of CDDP + VIT C and CDDP groups were decreased in day 2 of treatments. Food intakes of CDDP group was significantly lowers than others groups on day 3 and 4 of treatments (P<0.05). At day 4 of treatments, VIT C and CDDP + VIT C groups had significantly higher average food intakes while CDDP group had lower compared to before treatment.

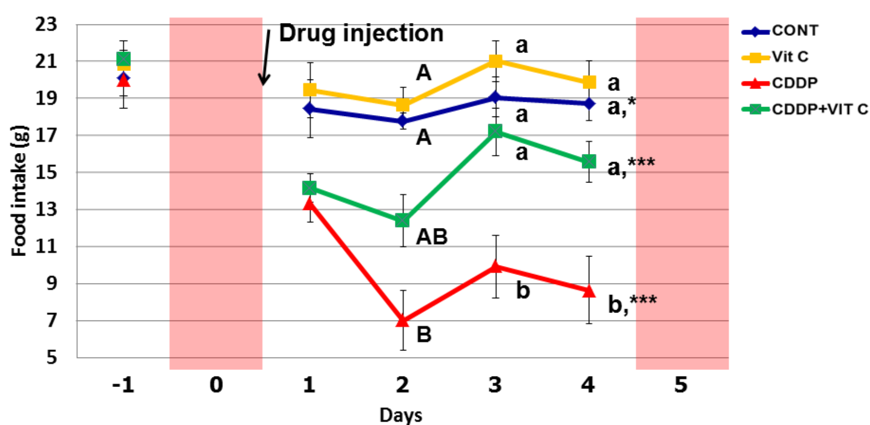

Figure 5. Daily mean values for food intake in four groups of rats throughout the experimental period.

Different superscripts (a, b) means differ significantly (p<0.05) using one-way ANOVA followed by Student Newman-Keuls method.

A, B Significant difference in non-parametric method (p<0.05).

*= P<0.05 and *** = P<0.001 significant difference from the values before treatment (day -1) using paired t-test.

The shade area showed rats in metabolic cage.

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Water intakes were similar among groups throughout the experiments as shown in figure 6.

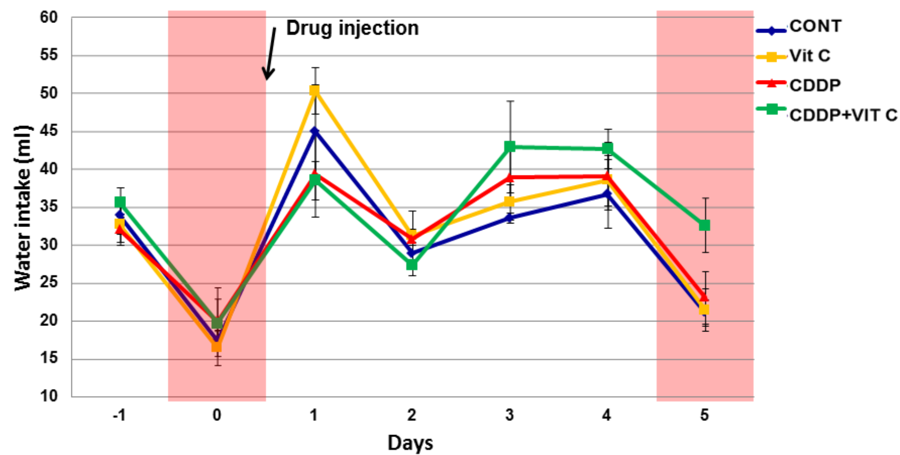

Figure 6. Daily mean values for water intake in four groups of rats throughout the experimental period.

The shade area showed rats in metabolic cage.

Systolic arterial pressure, hematocrit, kidney biochemical porfiles, plasma electrolytes, UPC and urinary protein excretion

According to table 2, systolic arterial pressure (SAP) were similar among groups on both days before and after 5 days of treatments. After 6 days of treatments, hematocrit were significantly decreased by 8.5% ($p<0.001$), 5.2% ($p<0.05$), 4.3% ($p<0.005$) and 9.5% ($p<0.001$) in CONT, VIT C, CDDP and CDDP + VIT C groups compared with the pre-treatment value, respectively. However, the value of SAP and Hct of all groups in both before and after 6 days of treatments were still in the normal range.

The levels of P_{cr} and PUN were considered as serum biochemical markers for renal function. In CDDP and CDDP + VIT C groups, the value of P_{cr} and PUN were

significantly higher than other groups and were highest in CDDP group which received cisplatin alone. In CDDP group, P_{cr} and PUN were significantiy increased by 832% ($p<0.001$) and 687% ($p<0.001$) after 6 days of treatments compared with before treatments respectively. In CDDP + VIT C group, P_{cr} and PUN were significantiy increased by 262% ($p<0.001$) and 148% ($p<0.001$) after 6 days of treatments compared with the pre-treatment value. GFR were significantly decreased in CDDP and CDDP + VIT C groups by 82.5% ($p<0.001$) and 70.3% ($p<0.001$) after 6 days of treatments as compared with the pre-treatment value respectively. Although the use of high dose vitamin C combined with CDDP can diminished CDDP-induced renal damage by increasing GFR and decreasing P_{cr} and PUN levels compared with CDDP treatments alone, the value were still significantiy different from non-CDDP treatments groups. Urine volume of CDDP + VIT C groups was higher significantly compared with groups without CDDP ($P<0.05$).

Plasma Na^{+} concentrations of CDDP group was slightly lower than other groups but it was still in normal range. Plasma concentrations of K^{+} and Cl^{-} were similar and fell within normal range among groups.

The urinary protein to creatinine ratio (UPC) after 6 days of CDDP administration was significantiy higher in CDDP and in CDDP + VIT C groups compared to before treatment ($p<0.001$) and compared with group without CDDP ($P<0.05$). The urinary protein excretion was changed in the same pattern as UPC ratio.

Table 2. Systolic arterial pressure, hematocrit, kidney biochemical porfiles, GFR, plasma electrolytes, and urinary protein excretion before and at 6 days of treatments.

Parameters		Groups			
		CONT (n=9)	VIT C (n=9)	CDDP (n=11)	CDDP + VIT C (n=16)
SAP (mmHg)	D0	93.14 ± 1.92	95.17 ± 5.61	104.9 ± 2.6	106.1 ± 2.9
	D6	110.4 ± 2.6	103.4 ± 2.6	99.30 ± 5.94	103.5 ± 3.5
Hct (%)	D0	49.89 ± 0.46	49.83 ± 0.60	49.46 ± 0.49	50.13 ± 0.59
	D6	45.67 ± 0.60***	47.22 ± 0.91*	47.32 ± 0.75**	45.38 ± 0.60***
P_{cr} (mg %)	D0	0.500 ± 0.024	0.478 ± 0.032	0.536 ± 0.020	0.469 ± 0.018
	D6	0.533 ± 0.017^{A}	0.467 ± 0.017^{A}	5.200 ± 1.248^{B,†††}	1.700 ± 0.193^{B,***}
PUN (mg %)	D0	21.78 1.49	21.00 ± 1.38	19.69 ± 2.11	23.19 ± 2.00
	D6	17.53 ± 1.41^{A,*}	16.78 ± 1.06^{A}	154.9 ± 33.8^{B,†††}	57.55 ± 6.47^{B,***}
GFR (µl/g/min)	D0	2.439 ± 0.236	2.561 ± 0.251	2.292 ± 0.138	2.886 ± 0.244
	D6	2.582 ± 0.225^{A}	3.184 ± 0.228^{B}	0.472 ± 0.159^{C,***}	0.964 ± 0.116^{D,***}
U_{Vol} (µl/g/min)	D0	0.017 ± 0.002	0.017 ± 0.003	0.019 ± 0.003	0.021 ± 0.022
	D6	0.021 ± 0.003^{A}	0.027 ± 0.005^{A}	0.038 ± 0.005^{AB,†}	0.054 ± 0.024^{B,†††}
P_{Na+} (mEq/l)	D6	145.7 ± 0.6^{A}	144.4 ± 0.7^{AB}	142.8 ± 1.3^{B}	143.6 ± 0.7^{AB}
P_{K+} (mEq/l)	D6	6.367 ± 0.279	6.278 ± 0.201	6.064 ± 0.474	6.456 ± 0.267
P_{Cl-} (mEq/l)	D6	112.4 ± 3.0	114.8 ± 0.8	112.8 ± 5.0	117.1 ± 1.2
UPC (mg/mgCr)	D0	2.234 ± 0.159	1.897 ± 0.257	1.939 ± 0.192	1.949 ± 0.160
	D6	1.742 ± 0.212^{A,*}	1.457 ± 0.201^{A,*}	6.754 ± 1.008^{B,***}	10.15 ± 0.53^{B,***}
U_{Prot}*V (µg/g/min)	D0	0.026 ± 0.001	0.022 ± 0.003	0.024 ± 0.010	0.027 ± 0.003
	D6	0.023 ± 0.003^{AB}	0.021 ± 0.003^{A}	0.072 ± 0.012^{BC,**}	0.137 ± 0.005^{C,***}

Abbreviations: P_{e-}, Plasma concentration of electrolyte; U_{Vol}, Urine volume; U_{Prot}*V, Urinary protein excretion; UPC, Urinary protein to creatinine ratio.

The data were show as mean ± SE

Different superscripts (a, b) and (A, B) indicate statistically significance among groups using one-way ANOVA and one way ANOVA on rank ($p<0.05$).

*= $P<0.05$, **=$P<0.01$ and *** = $P<0.001$ significant difference from the values before treatment using paired t-test. ††† = $P<0.001$ significant difference from the values before treatment using Wilcoxon signed rank test.

Fractional excretion (Na^+,K^+,Cl^-), osmolarity clearance (Cosm) and free water clearance (CH_2O)

Fractional excretion of electrolytes (FE Na^+, FE K^+, FE Cl^-), osmolarity clearance (Cosm) and free water clearance (CH_2O) at 5 days of treatment were presented in table 3. The values of FE Na^+, FE K^+ and FE Cl^- of CDDP and CDDP + VIT C were significantly higher than CONT and VIT C groups ($p<0.05$). The value was lower in group receiving vitamin C compared with in group receiving CDDP alone.

The levels of Cosm in CDDP + VIT C group was significantly higher than other groups ($p<0.05$). The value of CH_2O was not significantly different among groups although the CH_2O tended to be higher in CDDP group.

Table 3. Fractional excretion (Na^+,K^+,Cl^-), osmolarity clearance (Cosm) and free water clearance (CH_2O) at 5 days of treatments.

Parameters	Groups			
	CONT (n=9)	VIT C (n=9)	CDDP (n=11)	CDDP + VIT C (n=16)
FE Na^+ (%)	0.734 ± 0.071^A	0.771 ± 0.075^A	17.29 ± 6.07^B	4.216 ± 0.655^B
FE K^+ (%)	11.85 ± 1.16^A	11.51 ± 1.19^A	160.2 ± 37.5^B	62.39 ± 9.35^B
FE Cl^- (%)	0.272 ± 0.032^A	0.317 ± 0.052^A	14.67 ± 6.19^B	2.133 ± 0.392^B
Cosm (µl/g/min)	0.065 ± 0.005^a	0.072 ± 0.006^a	0.065 ± 0.008^a	0.095 ± 0.007^b
CH_2O (µl/g/min)	-0.044 ± 0.005	-0.045 ± 0.003	-0.028 ± 0.006	-0.042 ± 0.008

The data were show as mean ± SE

Different superscripts (a, b) and (A,B) indicate statistically significance using one-way ANOVA and one-way ANOVA on rank. ($p<0.05$)

Oxidative stress markers in plasma, urine and kidney

Oxidative stress markers in plasma and urine including malondialdehyde (MDA), total antioxidant status (TAS) and protein carbonyl (PC) concentrations were shown in table 4. The value of plasma MDA concentration was highest in CDDP group while plasma TAS level was lowest in CDDP + VIT C group ($p<0.05$). Plasma PC concentration was similar among group.

Urinary MDA/Cr in CDDP and CDDP + VIT C were significantly higher compared with non-CDDP regimen groups ($p<0.05$). When comparing before CDDP treatment, the values in CDDP and CDDP + VIT C groups after 6 days of treatments were significantly increased by 125% ($p<0.05$) and 67% ($p<0.005$), respectively. CDDP group had lowest urinary TAS and was significantly lower than control group ($p<0.05$). In

CDDP and CDDP + VIT C groups, the values of urinary PC concentrations were significantly lower in CDDP and CDDP + VIT C groups compared to pretreatment values ($p<0.01$, $P<0.05$, respectively). Kidney MDA and CAT levels were similar among groups. Kidney GSH (reduced glutathione) level in CDDP and CDDP + VIT C groups were significantly higher than CONT and VIT C groups ($p<0.05$).

Table 4. Oxidative stress markers in plasma, urine and kidney before and after 6 days of CDDP treatment.

Parameters		Groups			
		CONT (n=9)	VIT C (n=9)	CDDP (n=11)	CDDP + VIT C (n=16)
Plasma					
MDA (nmol/ml)	D6	3.341 ± 0.219^{A}	3.446 ± 0.310^{A}	6.511 ± 0.704^{B}	3.763 ± 0.289^{A}
TAS (AA%)	D6	23.39 ± 1.24^{a}	23.53 ± 1.69^{a}	20.47 ± 2.25^{ab}	16.64 ± 1.66^{b}
PC (nmol/mg protein)	D6	1.462 ± 0.088	1.512 ± 0.088	1.559 ± 0.076	1.796 ± 0.125
Urinary					
MDA/Cr (nmol/mgCr)	D0	18.57 ± 1.57	19.56 ± 1.59	19.19 ± 2.03	16.87 ± 1.47
	D6	13.05 ± 1.36^{A,*}	14.78 ± 1.72^{A,*}	43.33 ± 7.76^{B,*}	28.16 ± 2.66^{B,**}
TAS (AA%)	D0	74.30 ± 1.40	73.19 ± 2.89	70.29 ± 3.33	70.21 ± 2.50
	D6	75.48 ± 1.57^{A}	65.66 ± 4.33^{AB}	58.52 ± 4.02^{B}	65.00 ± 3.04^{AB}
PC (nmol/mg protein)	D0	9.333 ± 1.363	9.822 ± 1.375	8.084 ± 0.738	9.889 ± 1.621
	D6	7.607 ± 0.939^{AB}	9.517 ± 1.145^{A}	6.589 ± 0.751^{AB,***}	5.515 ± 0.499^{B,*}
Kidney					
MDA (nmol/mg protein)	D6	0.672 ± 0.042	0.644 ± 0.026	0.683 ± 0.047	0.610 ± 0.047
GSH (µmol/mg protein)	D6	0.452 ± 0.030^{a}	0.481 ± 0.022^{a}	0.607 ± 0.028^{b}	0.612 ± 0.022^{b}
CAT (Unit/mg protein)	D6	203.6 ± 20.7	201.2 ± 23.3	184.8 ± 13.2	177.3 ± 6.9

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The data were show as mean ± SE

Different superscripts (a, b; A,B) indicate statistically significance using one-way ANOVA and one way ANOVA on rank, respectively ($P<0.05$).

*= $P<0.05$,**=$P<0.01$ and ***=$P<0.001$ significant difference from the values before treatment using paired t-test.

The relationships between renal funcions parameters and other parameters.

The polynomial relationships between GFR and other renal function and oxidative parameters in plasma and urine were investigated and showed in table 5. GFR had negative relationships with P_{cr} and PUN (figure 7). Moreover, the relationships were found between GFR and FE Na^+, FE K^+ and FE Cl^- (figure 8). GFR also had positive relationship with Cosm and negative relationship with CH_2O. For oxidative stress markers in the plasma, the GFR had negative relationships with only plasma MDA (figure 9A) but not for PC and TAS. In the urine, GFR had negative relationship with urinary MDA/cr and urinary TAS (figure 9B and 9C, respectively) but not PC.

Table 6 showed the relationships between GFR and oxidative stress markers in the kidney and body weight using simple linear regression analysis. Only, kidney GSH had positive linear regression with GFR (figure 10A). No relationship between GFR and kidney catalase and MDA was found. The urinary protein creatinine ratio was related to GFR (figure 10B). Moreover, GFR had negative relationship with body weight.

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Table 5. The relationships between GFR and other parameters using polynomial regression equation (n=45).

Parameters	Formular	P value	R
P_{cr}	P_{cr} = 0.5659+(0.957/GFR)+(-0.0206/GFR^2)	P<0.001	0.7566
PUN	PUN = 14.666+(31.5409/GFR)-(0.6589/GFR^2)	P<0.001	0.9121
FE Na^+	FE Na^+ = -1.3754+(4.2588/GFR)-(0.0745/GFR^2)	P<0.001	0.9863
FE K^+	FE K^+ = 12.0258+(31.2572/GFR)-(0.5549/GFR^2)	P<0.001	0.9752
FE Cl^-	FE Cl^- = -25.966+(4.0679/GFR)-(0.0715/GFR^2)	P<0.001	0.9628
C_{osm}	C_{osm} = 0.0831-(0.0028/GFR)+(0.0000026/GFR^2)	P<0.05	0.4260
C_{H2O}	C_{H2O} = -0.045+(0.0037/GFR)-(0.0000074/GFR^2)	P<0.001	0.6542
Plasma MDA	P_{MDA} = 3.6723+(0.3295/GFR)-(0.005/GFR^2)	P<0.001	0.5414
Plasma TAS	P_{TAS} = 19.3176+(0.5201/GFR)-(0.007/GFR^2)	P=0.2137	0.2662
Plasma PC	P_{PC} = 1.6518-(0.0223/GFR)+(0.0004/GFR^2)	P=0.6276	0.1481
Urinary MDA/Cr	$U_{MDA/Cr}$ = 15.9869+(6.2559/GFR)-(0.1267/GFR^2)	P<0.001	0.7999
Urinary TAS	U_{TAS} = 70.4404-(2.8829/GFR)+(0.0575/GFR^2)	P<0.001	0.5345
Urinary PC	U_{PC} = 7.2643-(0.303/GFR)+(0.0118/GFR^2)	P=0.1335	0.3024

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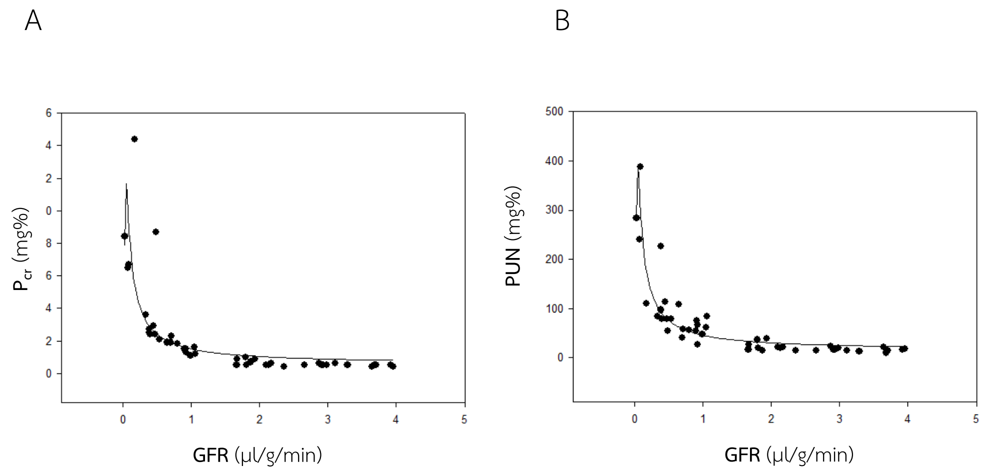

Figure 7. The relationships of GFR with P_{cr} (A) and PUN (B).

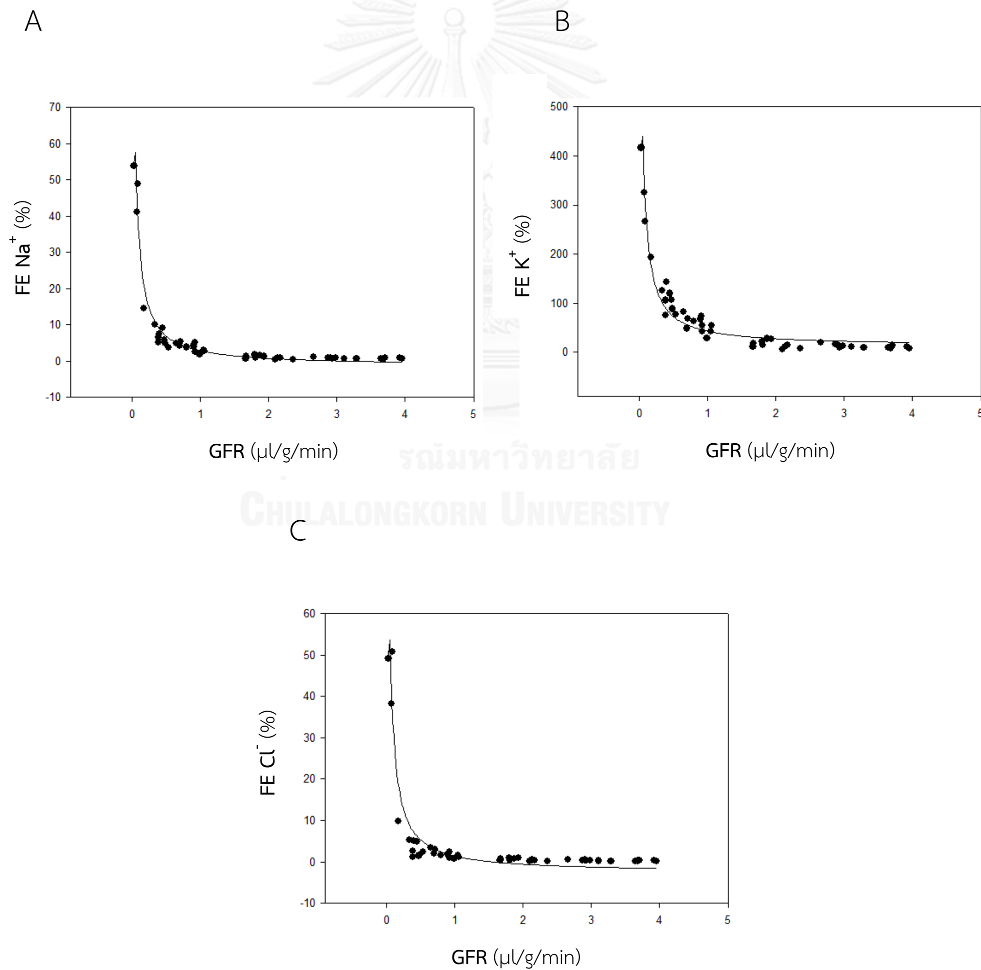

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Figure 8. The relationships between GFR and fractional excretions of Na^+ (A), K^+ (B) and Cl^- (C) using polynomial regression equation.

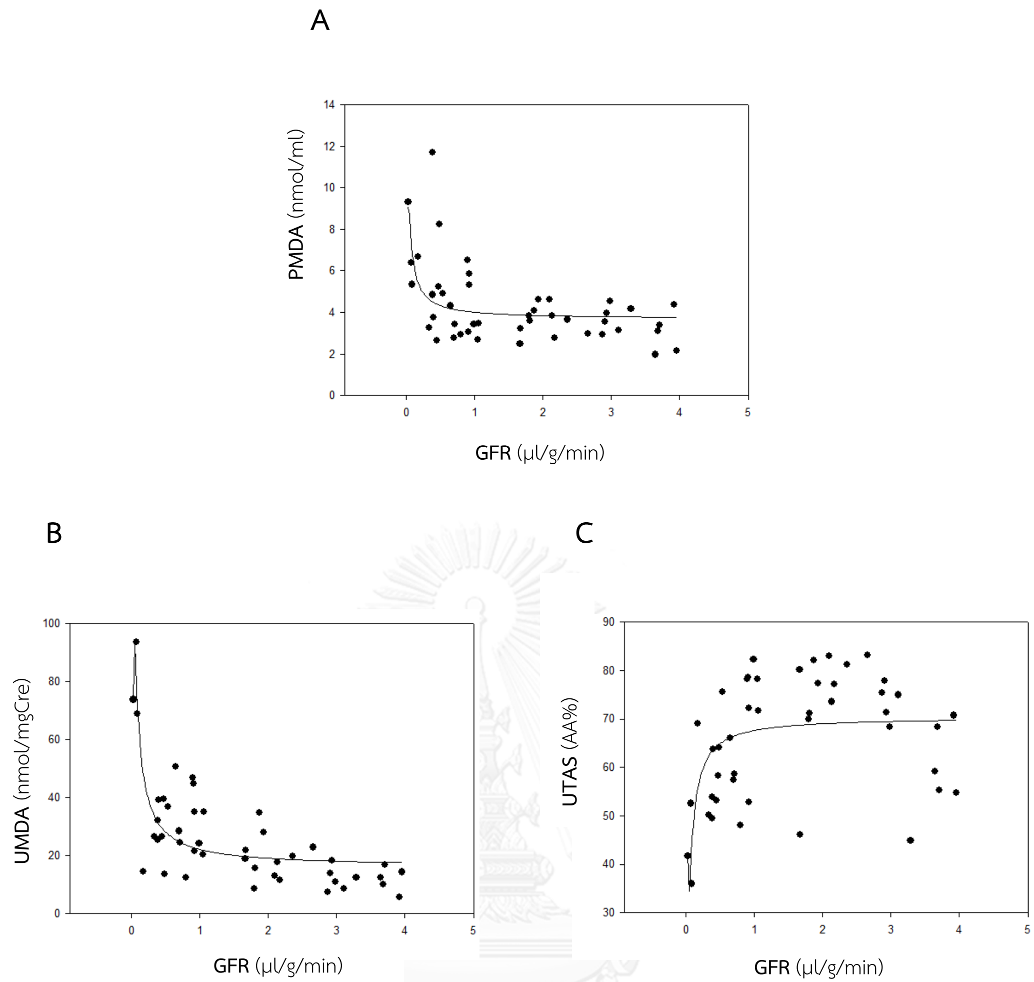

Figure 9. The relationships between GFR and oxidative stress markers including plasma MDA (A), urinary MDA (B) and urinary TAS (C).

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Table 6. The relationships between GFR and other parameters using simple linear regression equation (n=45).

Parameters	Formular	P value	R
Kidney MDA	K_{MDA} = 0.641+(0.00403*GFR)	P=0.931	0.0327
Kidney GSH	K_{GSH} = 0.634+(0.0502*GFR)	P<0.001	0.556
Kidney CAT	K_{CAT} = 178.291+(6.751*GFR)	P=0.272	0.167
UPC	UPC = 10.215+(2.678*GFR)	P<0.001	0.757
Body weight	BW = 314.491+(6.015*GFR)	P<0.05	0.345

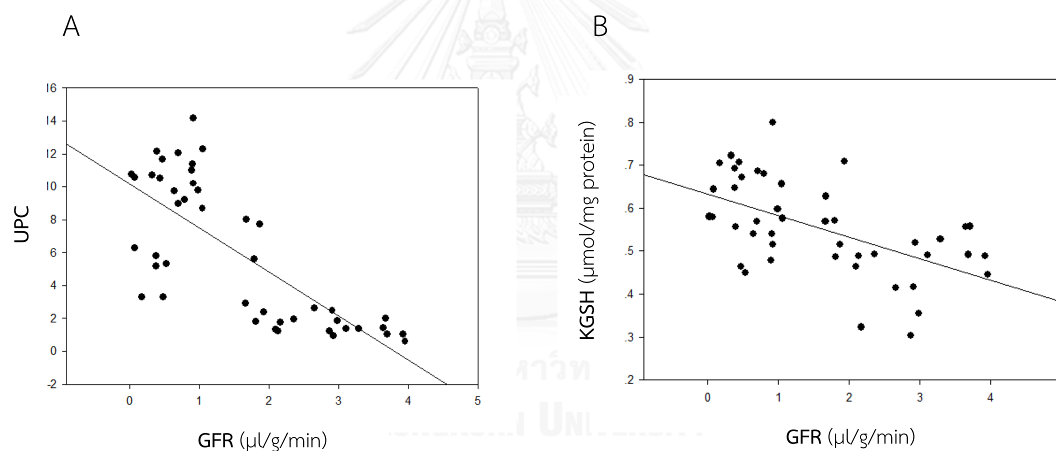

Figure 10. The relationships of GFR with UPC (A) and kidney GSH (B).

Gene expression of Bcl-2 (anti-apoptotic reguratory gene), Bax (apoptotic reguratory gene) and the ratio of Bcl-2 to Bax

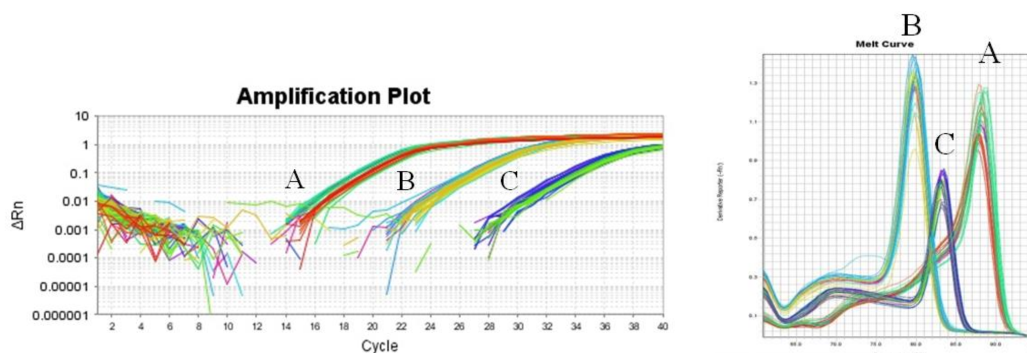

Figure 11. Amplifaction curves (left panal) and Melt curves (right panal) of mRNA quantification of GAPDH (A), Bcl-2 (B) and Bax (C) in 10 rats showed homogeneity of PCR product.

The amplifiation curves and melt curves of GAPDH, Bcl-2 and Bax were demonstrated as representative of each gene expression from CONT, VIT C, CDDP and CDDP + VIT C groups (figure 11). The relative mRNA levels of Bcl-2 and Bax at 6 days of treatment in both control and experimental groups were shown in figure 12A and 12B, respectively. The relative mRNA levels of Bcl-2 tended to be lower while the relative mRNA levels of Bax were higher in groups treated with CDDP alone or in combination with vitamin C. When calculating the ratio of Bcl-2 to Bax in CDDP and CDDP + VIT C groups, They were higher significantly compared with CONT group and group receiving vitamin C ($p<0.05$) (figure 12C). Vitamin C had no effect when given in both normal rats or rats treated with CDDP.

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The agorose gel electrophoresis patterns confirmd the correct size of realtime PCR preducts of Bcl-2, Bax and internal standard, GAPDH were shown in Figure 13A

and 13B. The samples of GAPDH products of PCR in the kidney of control rats was shown with molecular size of 309 (figure 13A) while two samples in rats receiving CDDP and CDDP + VIT C were run for Bcl-2 and Bax products as shown in figure 11B with molecular sizes near 62 and 97, respectively.

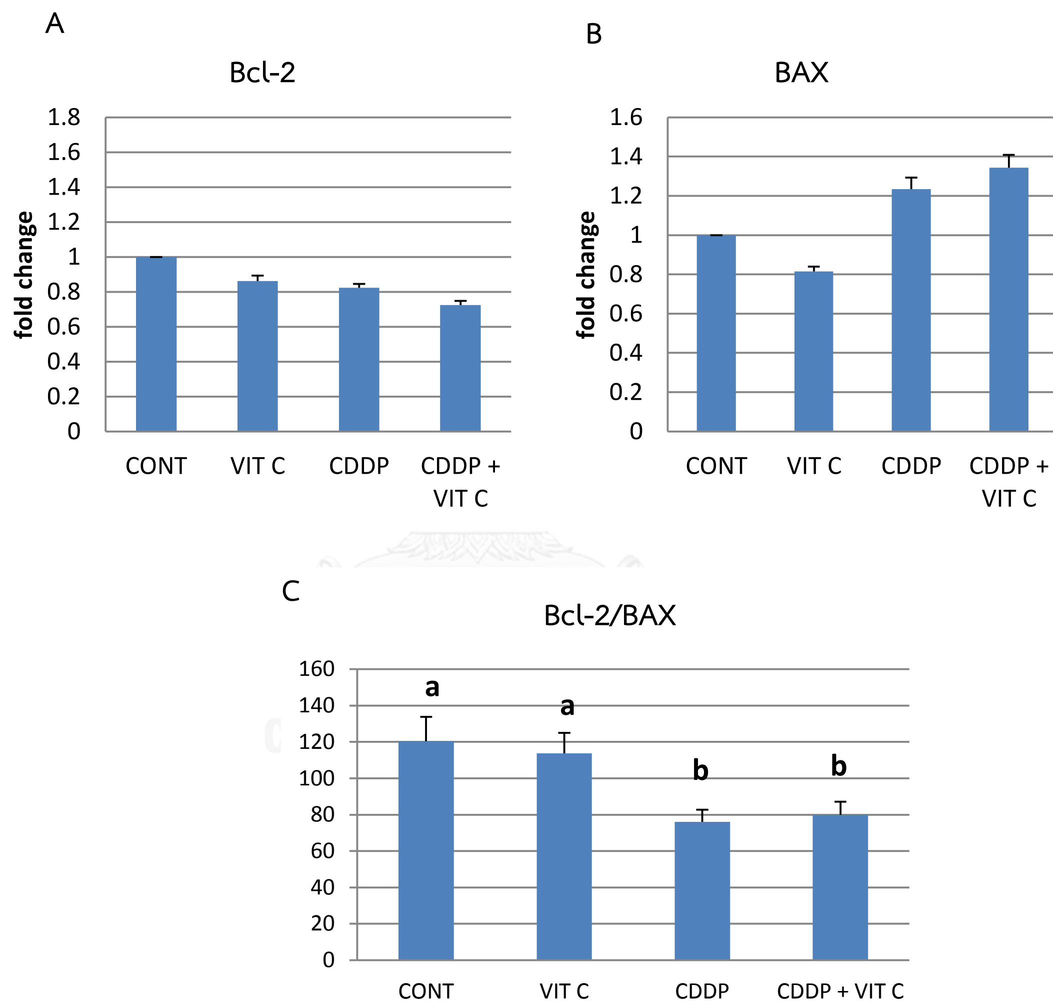

Figure 12. Relative mRNA levels of Bcl-2 (A), Bax (B) and the ratio of Bcl-2 to Bax mRNA expression (C)

Different superscripts means differ significantly ($p<0.05$) among groups on day 6 of CDDP treatment using one-way ANOVA.

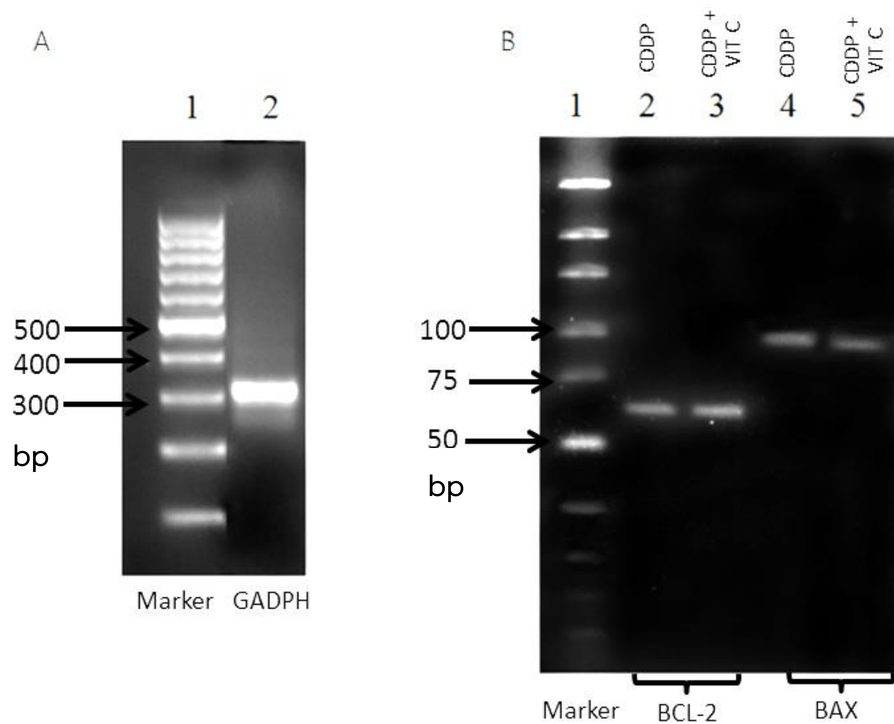

Figure 13. Agarose gel electrophoretic analysis of realtime PCR (RT-PCR) products. A ; 100 bp ladder molecular size marker and GAPDH products of RT-PCR were loaded in lanes 1 and 2, respectively. B ; 10 - 300 bp ladder molecular size marker was loaded in lanes 1. Two samples in CDDP and CDDP+vitC groups were run for Bcl-2 products of RT-PCR in lanes 2 and 3 and for Bax products of RT-PCR in lanes 4 and 5.

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CHAPTER V
DISCUSSION

Cisplatin toxicity can be generally monitored by a change in body weight because of decreased food ingestion. In the current study, the results showed that the body weight and food intake were significantly decreased in CDDP group. These finding were in agreement with previous reports (Humanes et al., 2012; Rastghalam et al., 2014). The results of body weight loss might be due to the sequential effects following renal impairment by the effect of CDDP. However, the food withdrawal in urine collection process may involve in the reduction of body weight.

The pathophysiology of CDDP nephrotoxicity is a complex process which can be grouped in four types of injury (Sanchez-Gonzalez et al., 2011). Firstly, tubular injury is the main targets of CDDP-induced renal damage. CDDP causes a direct injury to epithelial cells resulting in fluid and electrolytes disorders. The second process is vascular damage due to obstructive and/or inflammatory causes resulting in the reduction of renal blood flow. Glomerular injury is the third process, which is manifested by decreased GFR, increased plasma concentrations of creatinine, urea nitrogen and proteinuria. Lastly, CDDP can cause interstitial injury in long-term treatment, which may lead into chronic renal disease.

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In the present study, CDDP caused renal impairment in both glomerulus and tubular parts. A single dose of administration resulted in significantly decreased glomerular filtration rate by 82.5% and dramatically increased plasma concentrations of creatinine and urea nitrogen in CDDP group. CDDP also increased urinary protein excretion, which was demonstrated by increasing the values of both UPC and protein excretion. Thus, the glomerular target might involve the glomerular filtration barrier

which was reported previously by Abdelmeguid et al. (2010) that a single dose of CDDP (5 mg/kg) resulting in morphological changes of glomerulus including hypertrophied podocyte pedicels and thickened glomerular basement membrane at 14 days after the drug injection in male Sprague Dawley rats. Reduction in renal function was observed as early as 6 days after a single dose of CDDP at 6 mg kg^{-1}. These findings were in agreement with a report of Zhou et al. (2006) which injected CDDP at the dose of 6 mg/kg intraperitonealy into male Sprague Dawley rats and found the increased PUN and P_{cr} as early as 6 days after the drug injection. Moreover, renal functional loss was observed to reach the peak level at day 5, and then decreased to the basal level at day 14 after a single dose of CDDP at 6 mg/kg (Zhou et al., 2006). Previous reports suggested that the effect of CDDP-induced nephrotoxicity increased with dose and frequency of the drug administration (Madias and Harrington, 1978; Miller et al., 2010). In clinical usage, patient who received CDDP at a standard dose of 50 mg/m^2 body surface area might cause renal injury which were reported in patients receiving chemotherapy (Hartmann et al., 1999).

Rather than the glomerular changes, CDDP causes tubular cell damage. In the present study, CDDP administration increased urinary fractional excretion of sodium, potassium and chloride. Increased urinary excretion of electrolyte has been well documents to correlated with tubulointerstitial nephritis (Futrakul et al., 1999) and related to severity of chronic kidney disease in dog (Buranakarl et al., 2007). The impaired tubular reabsorptive capacity caused by CDDP was in agreement with previous reports (Wolfgang et al., 1994; Humanes et al., 2012; van Angelen et al., 2013). Humanes et al. (2012) observed that a single injection of CDDP (5mg/kg) in rats resulting in renal tubular injury and increased FE Na^+ on the fifth day after the drug

administration. Moreover, the tubular water reabsorption was also diminished in the present study as shown by increased renal water clearance. CDDP had a detrimental effect by tubular obstruction (Karimi et al., 2005), thus, the urine flow rate was increased which indicated the loss of reabsorptive capacity of both substances and water which might lead to chronic tubular disease.

Although proteinuria was presented after CDDP treatment, the values of SAP were similar between control and CDDP-treated groups. These results were similar to previous report from Bagnis et al. (2001) which showed no change in blood pressure at 9 days after CDDP (6mg/kg) injection in rats. However, it was not in agreement with Ali et al. (2011) who showed that rat receiving CDDP at the dose of 5 mg/kg had decreased blood pressure on day 5 after the drug treatment. Changes in renal function and blood pressure after CDDP were proposed to be mediated via angiotensin II (Saleh et al., 2009). Nonetheless, a nonpeptide angiotensin II (AII) receptor blocker, losartan, could not alter the onset or severity of cisplatin nephrotoxicity in rats after 6 days of 5 mg/kg single injection (Deegan et al., 1995) and also in rats receiving chronic administration of CDDP at the dose of 2.5 mg/kg daily for 7 days (Rastghalam et al., 2014).

It was noticed that vitamin C giving to normal rats had no effect on body weight, renal functions, proteinuria and blood pressure. However, giving vitamin C in rats receiving CDDP improved body weight and reduced nephrotoxicity although they were not normalized to control group. Intravenous megadose vitamin C can diminished the effect of CDDP on GFR and urinary excretion of electrolytes. However, megadose vitamin C injection could not ameliorated proteinuria induced by CDDP. Takano et al. (2002) previously suggested that CDDP decreases the receptor-

mediated endocytosis of protein would be the mechanisms underlying the proteinuria.

It has been known that nephrotoxicity is the major dose-limiting adverse effect of CDDP, primarily induced by inhibition of DNA synthesis (Pabla and Dong, 2008). Oxidative stress and apoptosis have been reported to play an important role in renal toxicity of platinum-based anticancer drugs (Siddik, 2003; Santos et al., 2007). CDDP promoted ROS generation and inhibited the activity of antioxidant enzyme resulting in oxidative stress (Pabla and Dong, 2008; Miller et al., 2010). In this study, the concentration of MDA was used to determine lipid peroxidation in plasma, urine and kidney tissue. The results showed increased MDA levels in plasma of CDDP group two times higher comparing to others groups without the alteration in plasma PC levels. The increments of urinary MDA levels were also found at 6 days after CDDP injection. The renal GSH levels were elevated in CDDP group while kidney CAT levels were relatively depleted in CDDP-treated group compared to the CONT group. The reduction in the renal glutathione level has been observed in rats in response to CDDP-induced oxidative stress (Fujieda et al., 2011). In contrast, Tian et al. (1997) observed that there might be positive regulation in the glutathione biosynthesis under oxidative stress conditions, resulting in increased glutathione levels. Since the CDDP mediated the oxidative stress leading to renal impairment, high dose vitamin C was used to counteract the effects of CDDP.

The renal oxidative stress induced by CDDP could be attenuated by vitamin C treatment. Vitamin C is one of the most frequently used antioxidant against CDDP-induced nephrotoxicity (Antunes et al., 2000; Fatima et al., 2007). However, vitamin C can act as a pro-oxidant depending on dosage and route of administration (Chen et

al., 2007; Chen et al., 2008). These results supported that the dosage and the manner of vitamin C administration in this study promoted antioxidant activity of vitamin c against CDDP-induced renal oxidative stress. The value of urinary TAS in CDDP + VIT C group was increased compared to CDDP group. However, plasma TAS was unaltered in CDDP group which might be due to CDDP affects primarily in the kidney much more than in other organs while the reduction of plasma TAS in CDDP + VIT C group may result from the alteration in plasma redox status caused by megadose vitamin C. Previous study described that megadose vitamin C promoted the production of ROS via superoxide-driven Fenton reaction *in vitro* (Michels and Frei, 2013). For the *in vivo* study, Chen et al. (2007) suggested that the ROS formation following intravenous administration of 0.5 g/kg of vitamin C in rats might be attenuated by intravascular antioxidants. The product of oxidative damage, both plasma and urinary MDA were lower in group receiving vitamin C and CDDP comparing with CDDP alone.

The nephrotoxicity caused by CDDP was in part mediated via oxidative damage which could be ameliorated using megadose vitamin C. The present study demonstrated the relationships between oxidative stress parameter and renal function, GFR. The results showed the negative non-linear relationships of P_{cr}, PUN, FE Na^+, FE K^+, FE Cl^-, CH_2O, plasma and urinary MDA and urinary TAS with GFR. The high oxidative damage was dramatic when renal impairment was severely diminished. Higher marker levels in the blood and urine might be in part related to reduction in urinary excretion. However, the results support the fact that the detrimental effect of glomerular filtration was related to oxidative damage.

Although free radicals generation has an important role in the mechanism of CDDP-induced nephrotoxicity, other factors may be contributed to CDDP induced cell death. By looking mRNA expression for the apoptosis markers, the ratio of anti-apoptotic level (Bcl-2) to pro-apoptotic level (Bax) appears to function as a rheostat that determines the survival or death by apoptosis in cells (Sheikh-Hamad et al., 2004). In the present study, Bcl-2 decreased while Bax increased although not significant resulting in significant lower Bcl-2/Bax in CDDP group. There was a report of CDDP induced apotosis in the study by Sheikh-Hamad et al. (2004), which demonstrated that administration of CDDP (5 mg/kg) significantly increased the levels of Bax expression but did not alter the expression of Bcl-2 in rat kidney at 5 days after the drug injection resulting in the declination of Bcl-2/Bax mRNA ratio promoted apoptosis.

Althoug vitamin C could improve renal function compared with rats receiving CDDP alone and the level of Bcl-2/Bax was still maintained in low level. Thus, a single administration of megadose vitamin C could not alter the effect of CDDP-induced apoptosis, which was not corresponding to the renal functional improvement. Therefore, the mechanism of CDDP induced nephrotoxicity may mediated directly at DNA level and may involve other mechanism such as oxidative damage. Moreover, the administration of megadose vitamin C alone to normal rats did not alter body weight, plasma creatinine, PUN, proteinuria, blood pressure, oxidative status and apoptosis markers.

In conclusion, a single administration of megadose vitamin C (5 mg/kg) injected at the time of CDDP administration can protect against CDDP-induced alterations of renal function in both GFR and renal electrolyte handling. The

mechanism is mediated via anti-oxidative damage. However, the mechanisms rather than oxidative stress may play roles in proteinuria induced by CDDP since it could not be prevented by vitamin C. Higher renal apoptosis was seen both in CDDP and CDDP plus VIT C groups. Thus, reduction in renal oxidative damage caused by megadose vitamin C may improve renal function while the apoptotic process was still maintained.

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จุฬาลงกรณ์มหาวิทยาลัย
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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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VITA

Miss Supa Sithanukul was born on May 31st, 1985 in Bangkok, Thailand. She received DVM from Faculty of Veterinary Science, Chulalongkorn University in 2010 with 2nd class of honor. She proceeds her study in Master of Science Program in Animal Physiology, Faculty of Veterinary Science, Chulalongkorn University. Her interest is in nephrology.

LIST OF PUBLICATION

1. Sithanukul S, Benjanirut C, Buranakarl C 2014. Renal Functions and Oxidative Stress in Rats Receiving Cisplatin and Megadose Vitamin C. Proceeding of the 8th VPAT Regional Veterinary Congress 2014 (VRVC 2014). May 18-22, 2014, Bangkok, Thailand.

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