Lipid Peroxidation and Renal Injury in Renal Ischemic Reperfusion: Effect of Angiotensin Inhibition

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Objectives: *To investigate the role of angiotensin inhibition on lipid peroxidation (LPO) and renal pathology in ischemic reperfusion (IR).*
Material and Method: *Male Wistar rats were subjected to 15-, 30-, 45- or 60- minutes of renal ischemia (I) by left renal artery occlusion. In the 30-minute I group, reperfusion (R) for 1 day ($I_{30}R_1$) was performed in additional animals that had been treated with water, angiotensin converting enzyme inhibitor (ACEI; enalapril 5 mg/kg/d), or angiotensin receptor type 1 blocker (ARB; losartan 10 mg/kg/d) one day before I and were continued for 1 day after R. Renal tissue malondialdehyde (MDA), an indicator of LPO, was examined during I and IR periods. Renal pathology was also determined.*
Results: *During ischemia, renal tissue MDA levels were increased throughout the 60-minute ischemic period and was maximum at 30 minutes of ischemia ($p < 0.01$). Histological changes in 30-minutes I group showed slight tubular cell congestion and mild interstitial edema. One day after reperfusion, MDA levels were still elevated ($p < 0.01$) when compared with sham. Progression of renal pathology was observed after 1 day of reperfusion. Both ACEI and ARB could attenuate the heightened MDA levels ($p < 0.01$). IR-induced renal injury was markedly diminished by administration of ACEI as well as by ARB.*
Conclusion: *These results indicate that inhibition of angiotensin could reduce lipid peroxidation and ameliorate renal injury during IR condition.*

Keywords: *Lipid peroxidation, Renal injury, Renal ischemic reperfusion, Angiotensin inhibition*

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Clinical and experimental studies have provided evidence that reactive oxygen species (ROS), generated mainly in the reperfusion (R) period of renal ischemic reperfusion (IR), play a crucial factor in producing renal cellular injury[1-8]. ROS can enhance lipid peroxidation (LPO) of mitochondrial, lysosomal and plasma membranes, leading to alterations in both membrane structure and function[6,7]. Moreover, recent studies have demonstrated that, in IR, oxidative stress and generation of ROS occur not only in the IR period but, actually, commence during the ischemic (I) time[8,9].

Indeed, IR also causes an early increase in intrarenal angiotensin II (Ang II) level[10,11]. Of interest, angiotensin converting enzyme inhibitors (ACEI)[12-14] as well as Ang II receptor blockers (ARB)[10,15-17] could attenuate impaired renal function and pathological lesion in IR model. In this regard, recent studies have demonstrated that Ang II could stimulate intracellular formation of ROS in several tissues including the kidney[18-22]. There are little data regarding the relationship among Ang II, ROS, LPO, and renal injury in IR model. The present study was conducted to examine the role of Ang II on renal LPO status and renal pathology in an IR model.

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Material and Method

Animals

The present study was performed in male Wistar rats weighing 220 to 250 grams obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Nakhonpathom). The animals were housed in a well-ventilated room kept at 23 to 25 C with an automatic lighting schedule, which provided darkness from 8 p.m. to 6 a.m. The animals were given free access to standard laboratory chow and drinking solution as in the following experiment protocol. All animals were used once only.

Experimental procedures

After familiarization with the new housing for three days, the animals were weighed and blood samples were collected from tails for creatinine measurement in order to assess kidney function (less than 1 mg%).

The rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg body weight) to examine the maximal LPO time point during renal ischemia. Using aseptic technique, a midabdominal incision was made to expose the kidneys. Then, a unilateral left renal pedicle cross clamp was performed for 15, 30, 45 or 60 minutes with microvascular clamps for ischemic groups (I_{15}, I_{30}, I_{45}, and I_{60}; respectively, n = 8 in each group). After ischemia, blood samples as well as ischemic kidneys in each group were collected. The sham group comprised of rats that were operated on and only wiped left renal pedicle. As will be shown in the result part, rats in the I_{30} group had maximal LPO level. To determine the role of Ang II on LPO in the IR model, thus, additional animals in the I_{30} group were divided into three groups (n = 8 rats/group). In these three I_{30} groups, the clamps were released for reperfusion and the incision was closed. The rats were kept after reperfusion for 1 day and were labeled as $I_{30}R_1$ animals. All $I_{30}R_1$ rats were allowed to wake up and returned to a clean cage with free access to food but with three different kinds of drinking solution: 1) Water (only distilled water), 2) Water + ACEI (enalapril 5 mg/kg/day), or 3) Water + ARB (losartan 10 mg/kg/day). The drinking solutions were provided one day before the operation and were continued for one day after reperfusion. The $I_{30}R_1$ animals were then re-operated under anesthesia. Blood samples and $I_{30}R_1$ kidneys were collected. Serum samples were stored at -80 C until use for creatinine, and blood urea nitrogen (BUN) measurements by using ISE (ion selection electrode) indirect method (Model CX3, Beckman Instrument INC, Germany). Each renal tissue sample was divided for histological evaluation (fixed in 10% formaldehyde) and for malondialdehyde (MDA) measurement (frozen in liquid nitrogen and then stored at -80 C).

MDA Level measurement

Levels of MDA in the kidneys were determined as an indicator of LPO following a protocol previously described [23, 24]. The frozen renal tissues were weighed and homogenized in 1.15% KCl (1 g of renal tissue in 9 ml of KCl) for 1 minute. A 0.2 ml aliquot of the homogenate was added to a reaction mixture containing 0.2 ml of 8.1% sodium dodecyl sulfate (w/v), 1.5 ml of 20% acetic acid (v/v), 1.5 ml of 0.8% thiobarbituric acid (w/v), and 0.6 ml of distilled water. The samples were mixed vigorously and then heated in a water bath (Memmert, Germany) at 95 C for 60 minutes. After cooling with tap water, the samples were added with 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1, v/v), and then shaken vigorously (at least 1 minute). The mixtures were centrifuged (Euroscan, Japan) at 1200g for 15 minutes. The organic layer was removed and the absorbance at 532 nm was measured by spectrophotometer (BTS-320; Biosystems, Spain). A standard curve of malondialdehyde bis (dimethyl acetal, 99%) in water was prepared covering the concentration range of 0-200 nmol/ml[23,24]. The content of LPO was reported as nmole MDA per mg protein. Protein concentration was assessed by Biuret's method (Spectronic 401; Milton ROY, USA). Measurements of all samples were conducted in duplicate.

Morphologic evaluation

Renal tissue injury was assessed by three-blinded pathologists from tissue sections stained by the Periodic Acid-Schiff (PAS) reaction. Sections were scored in a semi-quantitative manner. The numerical scores[25] were indicated as follows:

0 = normal structure
1 = areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving less than 25% of cortical tubules
2 = similar changes involving greater than 25% but less than 50% of cortical tubules
3 = similar changes involving greater than 50% but less than 75% of cortical tubules
4 = similar changes involving greater than 75% of cortical tubules
5 = complete cortical tubules necrosis

Chemical agents

Sodium pentobarbital (Nembutal) was purchased from Sanofi, France. Enalapril was obtained from Biolab, Thailand. Losartan was derived from MSD, USA. Formaldehyde, thiobarbituric acid, and sodium dodecylsulfate were obtained from Sigma, USA. Malonaldehyde bis (dimethyl acetal, 99%) was purchased from Aldrich, USA. Acetic acid (100%), n-butanol, and pyridine were supplied from Merk, Germany.

Statistical analysis

The data were expressed as mean $\pm$ SD. One-way ANOVA followed by Bonferroni's t-test was used to analyze the MDA values and blood chemistry levels with $p < 0.05$ considered as being significant. Pathological scores were presented in descriptive statistics by measures of central tendency (Mode).

Results

Ischemic periods

During the ischemic period, MDA levels were progressively increased ($p < 0.01$) and were profoundly enhanced at 30 minutes (Sham = 1.41 $\pm$ 0.08, I_{15} = 3.12 $\pm$ 0.15 and I_{30} = 3.84 $\pm$ 0.17 nmol/mg protein, Fig. 1). At 45- and 60- minutes of ischemia, MDA levels declined slightly but were still higher ($p < 0.01$) than sham (3.08 $\pm$ 0.19 and 2.87 $\pm$ 0.14 nmol/mg protein, respectively).

Histological studies in renal tissue from sham and during ischemia are shown in Fig. 2A and 2B, respectively. The 30-minute ischemia group showed mild interstitial edema with some mononuclear leukocyte infiltration as well as slight tubular cell congestion. The pathological score was between 1 and 2.

Reperfusion periods

In the $I_{30}R_1$ group, one day after reperfusion MDA levels were still high compared with sham (Sham = 1.41 $\pm$ 0.08, $I_{30}R_1$ + Water = 3.15 $\pm$ 0.19 nmol/mg protein; $p < 0.01$, Fig. 3). The progression of renal pathology was obvious after 1 day of reperfusion (Fig. 4). IR induced moderate tubular dilatation, vacuolar degeneration and cast formation as well as mild brush border membrane loss (Fig. 4A). The pathologic score was increased to 3.

Effect of ACEI or ARB on LPO and renal pathology in $I_{30}R_1$ animals

ACEI and ARB could attenuate the heightened MDA levels ($I_{30}R_1$ + ACEI = 1.90 $\pm$ 0.12 and $I_{30}R_1$ + ARB = 1.81 $\pm$ 0.20 nmol/ mg protein; $p < 0.01$, Fig. 3). The renal pathological lesions were markedly lessened by either ACEI (Fig. 4B) or ARB administration (Fig. 4C). There were only slight tubular cell swelling and some vacuolization. The pathological scores were reduced to 1.

Metabolic parameters and renal function

During the ischemic periods, there were no differences in renal function among I_{15}, I_{30}, I_{45}, and I_{60} groups (data not shown). In $I_{30}R_1$ group, the levels of BUN, creatinine, creatinine clearance, and urine flow rate were comparable and did not significantly differ from sham (Table 1). These parameters were also not significantly altered by treatment with angiotensin inhibition agents (Table 1).

Discussion

The present study has demonstrated that lipid peroxidation (LPO) was elevated throughout the 60-minute of renal ischemic period. LPO was persistently high at 24 hours after the reperfusion period. Minimal renal pathology was observed during renal ischemia while markedly progressive renal lesions were noted in the reperfusion time. Treatment with ACEI as well as ARB could diminish increased MDA levels and

Table 1. Levels of BUN, creatinine, creatinine clearance, and urine flow rate in Sham, $I_{30}R_1$ + Water, $I_{30}R_1$ + ACEI, $I_{30}R_1$ + ARB rats

Group	BUN (mg%)	Cr. (mg%)	CCr (ml/min/100g BW)	Urine flow rate x 10^{-2}(ml/min)
Sham	20.17 $\pm$ 0.48	0.45 $\pm$ 0.03	0.74 $\pm$ 0.02	2.02 $\pm$ 0.09
$I_{30}R_1$ + Water	20.83 $\pm$ 0.87	0.47 $\pm$ 0.04	0.75 $\pm$ 0.04	1.85 $\pm$ 0.09
$I_{30}R_1$ + ACEI	18.67 $\pm$ 1.33	0.47 $\pm$ 0.03	0.63 $\pm$ 0.03	2.02 $\pm$ 0.15
$I_{30}R_1$ + ARB	21.33 $\pm$ 0.67	0.50 $\pm$ 0.05	0.75 $\pm$ 0.05	2.18 $\pm$ 0.09

I, ischemia; R, reperfusion; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor type 1 blocker; BUN, blood urea nitrogen; Cr, creatinine; CCr, creatinine clearance; n = 8/ group

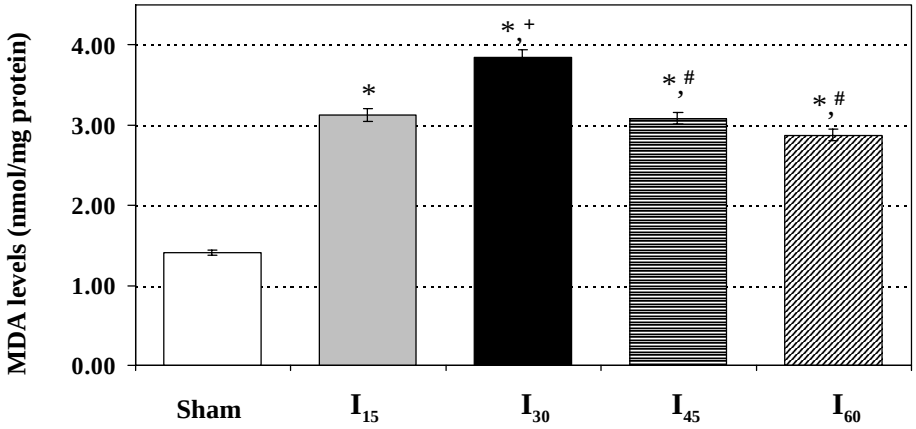

Fig. 1 Renal MDA level in Sham, I_{15}, I_{30}, I_{45}, and I_{60} (n = 8/group). * $p < 0.01$ vs control; $^{+}$ $p < 0.01$ vs I_{15}, $^{\#}$ $p < 0.01$ vs I_{30}

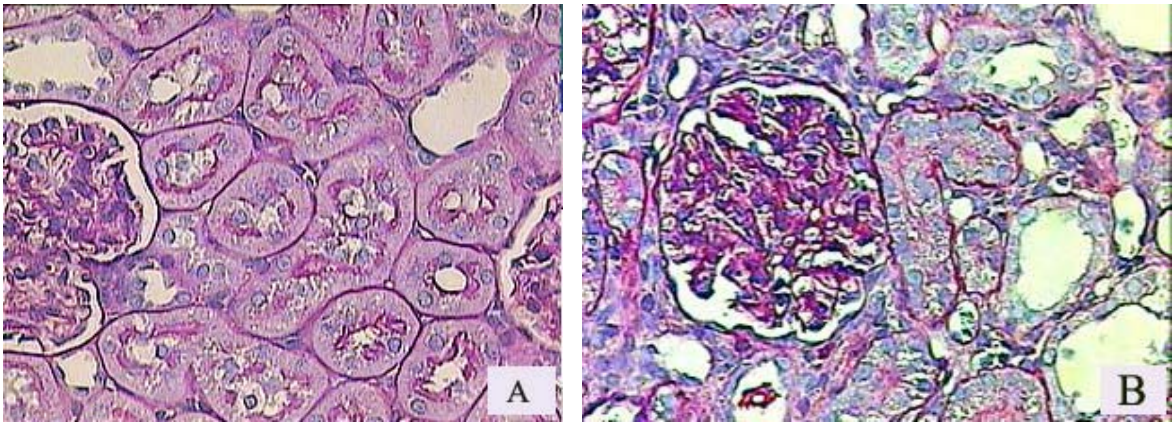

Fig. 2 Representative PAS-stained tissue sections of left renal cortex. A: Sham, and B: I_{30}. Original magnification: 200x

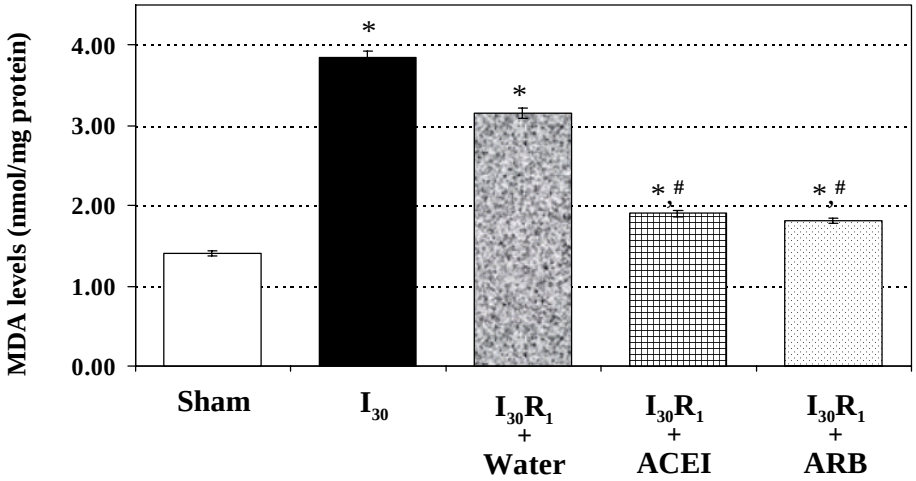

Fig. 3 Renal MDA level in Sham, I_{30}, $I_{30}R_1$ + Water, $I_{30}R_1$ + ACEI, and $I_{30}R_1$ + ARB (n = 8/group). * $p < 0.01$ vs control; $^{\#}$ $p < 0.01$ vs $I_{30}R_1$ + Water

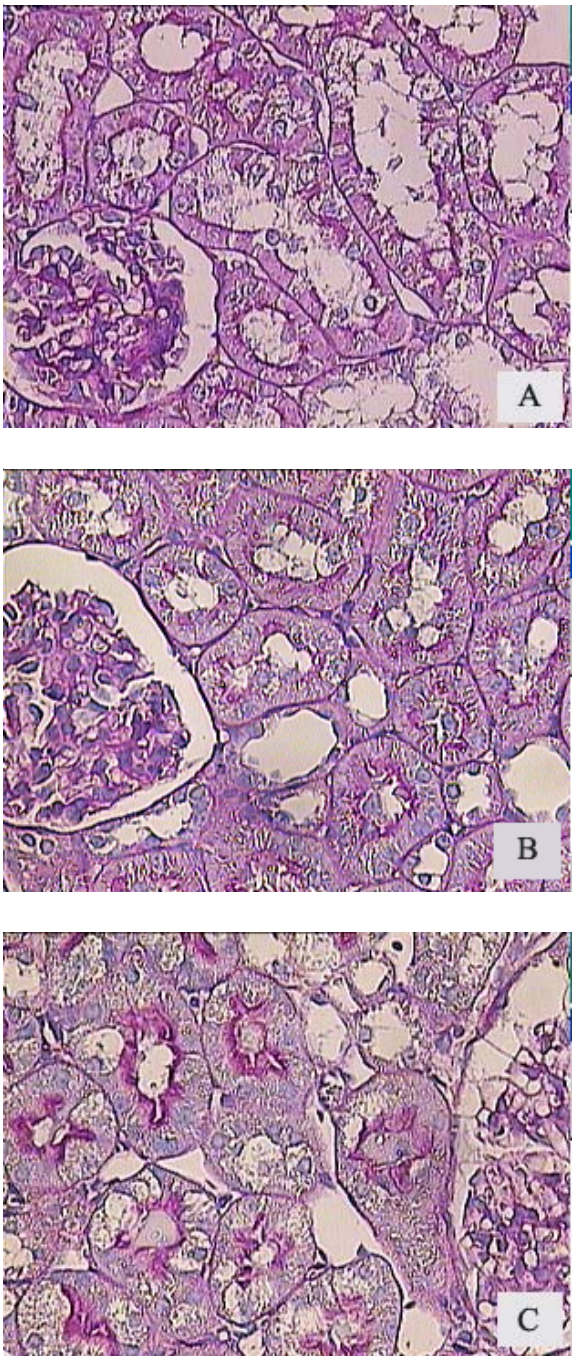

Fig. 4 Representative PAS-stained tissue sections of left renal cortex. A: $I_{30}R_1$ + Water, B: $I_{30}R_1$ + ACEI, and C: $I_{30}R_1$ + ARB. Original magnification: 200x

ameliorate renal pathology occurring in the reperfusion period.

ROS have been implicated as an important effecter in the IR-induced tissue damage of various organs including kidney[1-8]. Almost all previous studies in the IR model of the kidney have focused on the importance of the oxidative stress produced during reperfusion when a burst of ROS is generated after oxygen is reintroduced into the system after a period of ischemia[3-7]. As shown in the present study and only two earlier studies[8,9], LPO production in renal tissue can also be stimulated in the ischemic duration. Pathological lesions were not examined in both studies, though. In the present study, despite heightened LPO status occurring in the ischemic period, only minor renal histological lesions were identified by standard routine pathological examination (Fig. 2). Of interest, the elevated LPO levels without obvious renal pathological abnormalities during ischemia, observed in the present study, were also reported in other tissues[26,27]. It has been postulated that ROS generated in non-reoxygenated tissues may come from trace O_2 that has been originally present or leaked during anoxic condition[28,29]. Mitochondria appear to be the major intracellular source of ROS during ischemia[9].

As demonstrated in Fig. 3, MDA levels were persistently high after reperfusion. It is well-established that ROS generation is accelerated at the beginning of reperfusion[1-8]. Accumulation of hypoxanthine and xanthine oxidase occurring in the ischemic period plays an important role in ROS formation[1,2]. In agreement with earlier studies, histological lesions in renal tissues examined in the present study obviously supervene in reperfusion period[30,31]. In the present study, when LPO was decreased, renal pathology was markedly attenuated (Fig. 3, 4), indicating the role of LPO in inducing renal injury. Results from the present study; however, suggest that it might be necessary to have sustained LPO hyperactivity in order to cause a major deleterious effect in the kidney.

Previous studies have shown that IR could augment tissue Ang II levels in ischemic kidney at 24 hours following reperfusion[10,11]. Markedly improved renal function and renal pathology were observed in several models when ACEI, including captopril[32], enalaprilat[13], and saralasin[33], were administered prior to ischemic insult. Moreover, captopril and enalaprilat were shown to be effective whether given just prior to, at, or after reperfusion[14]. Treatment with losartan, an ARB, had also provided renoprotective effect in the reperfusion period[10,15-17]. However, the beneficial effects of ARB on renal damage seem to depend on the length of the ischemic period. Jerkic et al[34] demonstrated that losartan failed to reduce the damage of the kidney subjected to ischemia for 45 min. In the present

study, the authors performed ischemia only for 30 min because it is below the 45-min limit previously reported as the longest period of renal warm ischemia that allows reversible injury and compatible with survival[35]. The present study confirms that a 30-min ischemia is a safe period for reduction of renal injury in IR model.

ACEI and ARB have been proposed to protect against renal ischemic injury via lessening renal vascular resistance and increasing oxygen delivery and consumption[10-17]. Ang II inhibition would cause decreased renal vascular resistance by reducing activation of Ang II receptor type I and increasing synthesis of prostacyclin and nitric oxide[14]. Vasodilatation increases blood flow, thereby, providing the needed oxygen and substrate necessary for ATP synthesis and preventing cell death[14]. Some authors, however, have argued that, Ang II inhibition might actually worsen reperfusion injury by increasing the amount of potential oxygen radical-mediated injury via increased available oxygen[36]. In contradistinction with this latter contention, the present study has clearly illustrated that ACEI and ARB could diminish, rather than stimulate, renal LPO during the reperfusion period (Fig. 3). Moreover, recent studies have demonstrated that Ang II could increase intracellular formation of ROS in various tissues including the kidney[18-22]. The elevation of ROS is possibly mediated via activation of NADH/NAD(P)H oxidase by Ang II[20,21,37,38]. Thus, less ROS production would occur during Ang II inhibition. As such, the results in the present study have underscored the important role of Ang II, besides mitochondria and xanthine oxidase, in ROS generation in IR model.

In conclusion, renal ischemia enhanced LPO but induced only mild renal pathology. Following reperfusion, LPO was persistently high and major renal pathological changes were identified. ACEI as well as ARB could diminish LPO and ameliorate renal tissue injury during IR condition.

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ลิปิดเปอร์ออกซิเดชัน และการบาดเจ็บของไต ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว: ผลของการยับยั้งแองจิโอเทนซิน

อยู่เย็น ซื้อจ้าง, วิภาวี กิตติโกวิท, สมชาย เอี่ยมอ่อง, สมจิตร์ เอี่ยมอ่อง

วัตถุประสงค์: *เพื่อศึกษาบทบาทของการยับยั้งแองจิโอเทนซินต่อลิปิดเปอร์ออกซิเดชัน และการบาดเจ็บของไต ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว*

วัสดุและวิธีการ: *ทำการทดลองในหนูพันธุ์วิสต้า โดยทำการบีบหลอดเลือดแดงไตข้างซ้ายเป็นเวลา 15, 30, 45 หรือ 60 นาที (กลุ่ม I) ในกลุ่ม I 30 ให้ทำเพิ่มอีก แต่ให้ปลดการบีบหลอดเลือดแดง (R) หลังจากเย็บปิดแผลผ่าตัดแล้ว ทำการเลี้ยงสัตว์ทดลองต่อไปอีกเป็นเวลา 1 วัน เป็นกลุ่ม I 30R1 ซึ่งกลุ่มนี้จะถูกแบ่งออกเป็นอีก 3 กลุ่มย่อย คือ 1) ได้รับน้ำดื่มเพียงอย่างเดียว 2) ได้รับน้ำดื่มผสม angiotensin converting enzyme inhibitor (ACEI; Enalapril®; 5 mg/kg/d) และ 3) ได้รับน้ำดื่มผสม angiotensin II receptor type 1 blocker (ARB; Losartan®; 10 mg/kg/d) สัตว์ทดลองจะได้รับสารดังกล่าว 1 วันก่อนการผ่าตัดทำ I และรับต่อไปอีกเป็นเวลา 1 วัน หลังจากการทำ R เมื่อครบกำหนดการทดลอง ทำการเก็บตัวอย่างเนื้อไตเพื่อตรวจวัดระดับ malondialdehyde (MDA) ซึ่งเป็นตัวชี้วัดระดับลิปิดเปอร์ออกซิเดชัน รวมทั้งทำการตรวจวัดระดับการทำลายเนื้อไต*

ผลการศึกษา: *ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว ค่า MDA มีระดับเพิ่มขึ้นตลอดช่วง 60 นาที และมีค่าสูงสุด ณ ที่เวลา 30 นาที ($p < 0.01$) ซึ่งกลุ่ม I 30 นี้ มีการเปลี่ยนแปลงของเนื้อไต คือ เซลล์หลอดไตบวมเล็กน้อย และช่องว่างระหว่างเซลล์ขยายตัวปานกลาง ส่วนกลุ่ม I 30R1 ค่า MDA ยังคงมีระดับสูงเมื่อเปรียบเทียบกับกลุ่ม sham ($p < 0.01$) พบว่าภายหลังการทำ R เป็นเวลา 1 วัน มีการทำลายเนื้อไตมากขึ้น อย่างไรก็ตาม ACEI หรือ ARB สามารถลดทั้งระดับ MDA ที่สูง และระดับการทำลายเนื้อไต*

สรุป: *ผลการศึกษาครั้งนี้บ่งชี้ว่า การยับยั้งแองจิโอเทนซิน สามารถลดลิปิดเปอร์ออกซิเดชัน และการบาดเจ็บของไตในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว*
