# Chlorogenic Acid Ameliorates Vascular Remodeling and Perivascular Fibrosis in Kidney Fibrosis Model in Mice

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Abstract: Kidney Fibrosis is the common pathway final of Chronic Kidney Disease, which is characterized by vascular remodeling and perivascular fibrosis. The Unilateral Ureteral Obstruction (UUO) model is used to cause kidney fibrosis. Chlorogenic Acid (CGA) is an antioxidant as a renoprotective agent. However, fibrosis perivascular and vascular remodeling has not analyzed yet. Study has objective to examine the effect of CGA in vascular aspects, specifically vascular remodeling and perivascular fibrosis in kidney fibrosis. Material and Methods: This research was a true experimental study. Unilateral Ureteral Obstruction (UUO) was performed in swiss webster background mice (n=25, 2-3 months old, 20-30 g weight) to induce kidney fibrosis. The mice were divided into five groups, SO (Sham Operation/control), U7 (UUO day-7), U14 (UUO day-14), UC7 (UUO+CGA day-7), and UC14 (UUO+CGA day-14). CGA 14 mg/kg body weight/day was induced intraperitoneally. Vascular remodeling based on lumen area, mean wall thickness, and wall/lumen area ratio (WLAR) and perivascular fibrosis of intrarenal arteries were quantified using Sirius Red staining. Study found that UUO groups (U7 and U14) had significantly higher vascular remodeling, as shown by lower lumen area, higher mean wall thickness and higher WLAR, and perivascular fibrosis, as shown by higher area compared to SO group ($p<0.05$). On the other hand, CGA groups (UC7 and UC14) revealed lower vascular remodeling, as shown by higher lumen area, lower WLAR, and perivascular fibrosis, as shown by lower area significantly compared to UUO group ($p<0.05$). The mean wall thickness was lower, but the data was not significantly different. Study conclude that CGA ameliorates kidney fibrosis through vascular remodeling and perivascular fibrosis.

## 1 INTRODUCTION

Chronic Kidney Disease (CKD) is one of the world's public health priorities because of its rapid increase in the prevalence. Global Burden of Disease study in 2015, renal disease was the 12th leading cause of death, with 1.1 million deaths worldwide. In Indonesia, the high prevalence indicated by the enrollment of more than 15.000 new patients with CKD in health insurance in 2011, 87% were End-Stage Renal Disease (ESRD) patients (Perkumpulan Nefrologi Indonesia, 2011). Decreased kidney function and scar formation that occurs progressively will direct CKD to the disease course's final stage, ESRD (Mutsaers *et al*, 2015). Kidney fibrosis leads to the ESRD as a final common pathway to CKD (Fragiadaki & Mason, 2011). Kidney fibrosis is

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characterized by interstitial fibrosis, glomerulosclerosis, the formation and activation of myofibroblasts cells (Duffield, 2014).

Unilateral Ureteral Obstruction (UUO) is the most used experimental kidney fibrosis model, especially in studies related to irreversible Acute Kidney Injury (AKI) and CKD (Ucero *et al,* 2014). The changing of hemodynamic in CKD may induce vascular remodeling, which is influenced by activation of the renin-angiotensin system (RAS), endothelin-1 (ET-1), endothelial dysfunction, oxidative stress and asymmetric dimethylarginine (ADMA), and the anti-aging molecule Klotho (Briet & Burns, 2012). Fibrosis also causes vascular remodeling characterized by interstitial remodeling and then vascular remodeling, resulting in reduced blood supply to the kidneys, causing kidney failure (Efstratiadis *et al,* 2009). Endothelin-1 (ET-1) and Endothelial Nitric Oxide Synthase (eNOS), which catalyzes the production of nitric oxide (NO) has a significant role in vascular function as vasoconstriction and vasodilatation agent (Schiffrin, 2012). It is becoming increasingly clear that an imbalance between these two mediators is a characteristic of endothelial dysfunction and is essential in vascular remodelling (Farris & Colvin, 2012).

Myofibroblasts are biomolecular markers and terminally differentiated cells found in non-pathological situations responsible for the synthesis and accumulation of interstitial extracellular matrix components during kidney fibrosis as the pathogenesis of CKD (Farris & Colvin, 2012). Perivascular fibroblasts and pericytes have previously been identified as the major contributors to the fibrosis then myofibroblast population in the kidney, especially in vascular area (Kramann & Humphreys, 2014).

Chlorogenic acid is a phenolic acid with vicinal hydroxyl groups on aromatic residues derived from cinnamic acid esterification, including caffeic, ferulic, and p-coumaric acids with quinic acid.

Much evidence has shown that chlorogenic acid has many biological characteristics, including antibacterial, antioxidant, and anticarcinogenic activities, especially hypoglycemic, hypolipidemic, and renoprotective effects (Santana-Gálvez *et al,* 2017). Chlorogenic acid can improve kidney function in 5/6 nephrectomy rats effectively due to its anti-oxidation and inhibiting accumulation of extracellular matrix (Lou *et al,* 2016).

None of the existing studies have provided overall effects from chlorogenic acid on fibrosis conditions in several other organs, especially from the molecular profibrotic and vasoactive substances. Therefore, this study was conducted to examine CGA's effect on vascular aspects, specifically vascular remodeling and perivascular fibrosis in kidney fibrosis.

# 2 MATERIALS AND METHODS

This research was a true experimental study using a post-test only controlled group design. Consists of control and treatment groups. This study has obtained permission from the Medical and Health Research Ethics Committee Faculty of Medicine, Universitas Tadulako, based on the Ethical Clearance certificate number C.0942/UN28.1.30/KL/2018 on February 26, 2018.

## 2.1 Unilateral Ureteral Obstruction

Swiss Webster mice (n=25, 2-3 months old, 20-30 g weight) were used for the experiments. Mice were housed in the Department of Anatomy, Faculty of Medicine, Universitas Tadulako in a cage with the light-dark cycle of 12:12 hour, food and water *ad libitum*. Unilateral Ureteral Obstruction (UUO) was performed to induce kidney fibrosis. Mice were anesthetized with Sodium Pentobarbital (0.1mL/10 g weight) injected intraperitoneally. The right flank's region was opened, and the right ureter was visualized then double ligated, after that cut between the ligation sides. Sham operation (SO) control group procedure was used the same procedure except for ligating and cutting the ureter, only for visualized.

## 2.2 Chlorogenic Acid Administration

Chlorogenic acid (Sigma-Aldrich C3878-1G) was done with intraperitoneally injection with a dose 14 mg/kg body weight/day. Mice were divided into 5 groups. The distribution for each group: sham operation (SO) group, was injected distilled water intraperitoneally for 14 days as a control; mice with UUO was injected distilled water intraperitoneally for 7 days, called group U7; mice with UUO was injected with chlorogenic acid for 7 days called UC7 group; mice with UUO was injected distilled water intraperitoneally for 14 days called group U14; mice with UUO was injected chlorogenic acid for 14 days group UC14. Mice were euthanized on days 8 and 15.

## 2.3 Kidney Harvesting

In this study, kidney harvesting mice were anesthetized with Sodium Pentobarbital (0.1mL/10 g weight) injected intraperitoneally after that abdomen and thorax were opened. Perfusion was done with 0.9 % NaCl from the left ventricle. Right kidney tissues were harvested, and the one-half side was fixated in Normal Buffer. Formalin for 24 h, and used for the paraffin-embedded tissue process.

## 2.4 Sirius Red Staining

Tissue slides in paraffin disk were cut in 4 mm thickness. Paraffin sections were deparaffinized with PBS washing. Tissue slides were given Sirius Red working solution to the entire surface of tissue for 1 hour. Afterward, the slides were soaked sequentially in 100% ethanol and xylene 3 times each, then mounted and incubated for 24 hours. Vascular remodeling assessment based on lumen area, mean wall thickness, wall area, and lumen area ratio/WLAR, while perivascular fibrosis is based on the difference between perivascular fibrosis and intrarenal arteries blood vessel area. Ten to fifteen intrarenal arteries with <50mm in diameter were captured and used for quantification using *ImageJ® software.*

## 2.5 Statistical Analysis

Data were analyzed using the IBM® SPSS® Statistics program. Data normality test was conducted using the Shapiro-Wilk test. The homogeneity was conducted using the Levene test, then the numerical test using the One-Way ANOVA test for normal data distribution and the Kruskal-Wallis test for abnormal data distribution.

# 3 RESULTS

Vascular Remodelling and Perivascular Fibrosis Area Unilateral Ureteral Obstruction (UUO) has known can induce vascular remodeling based on the quantification of Sirius Red staining (Figure 1A). Chlorogenic acid effects to ameliorate vascular remodeling that occurs in the condition of kidney fibrosis. Quantitative analysis of the lumen area showed lower significantly in the U7 and U14 groups ($p<0.05$) compared to the SO group, then higher significantly in the UC7 and UC14 groups ($p<0.05$) compared to the U7 and U14 groups (Figure 1B). Quantitative analysis of the mean wall thickness showed higher significantly in the U7 and U14 groups ($p<0.05$) compared to the SO group, then based on the means data of UC7 and UC14 groups were lower compared to the U7 and U14 groups (Figure 1C). Quantitative analysis on WLAR showed higher significantly in the U7 and U14 groups ($p<0.05$) compared to the SO group, then based on the means data of UC7 group was lower compared to the U7 group, whereas in the UC14 group ($p<0.05$) was lower significantly compared to the U14 group (Figure 1D).

Unilateral Ureteral Obstruction (UUO) has known to induce perivascular fibrosis based on the quantification of Sirius Red staining (Figure 1A). Chlorogenic acid ameliorated perivascular fibrosis that occurs in kidney fibrosis. Quantitative analysis in the area of perivascular fibrosis showed higher significantly in the U7 and U14 groups ($p<0.05$) compared to the SO group, but based on the means data of UC7 group was lower compared to the U7 group, whereas in the UC14 group ($p<0.05$) lower significantly compared to the U14 group (Figure 1E).

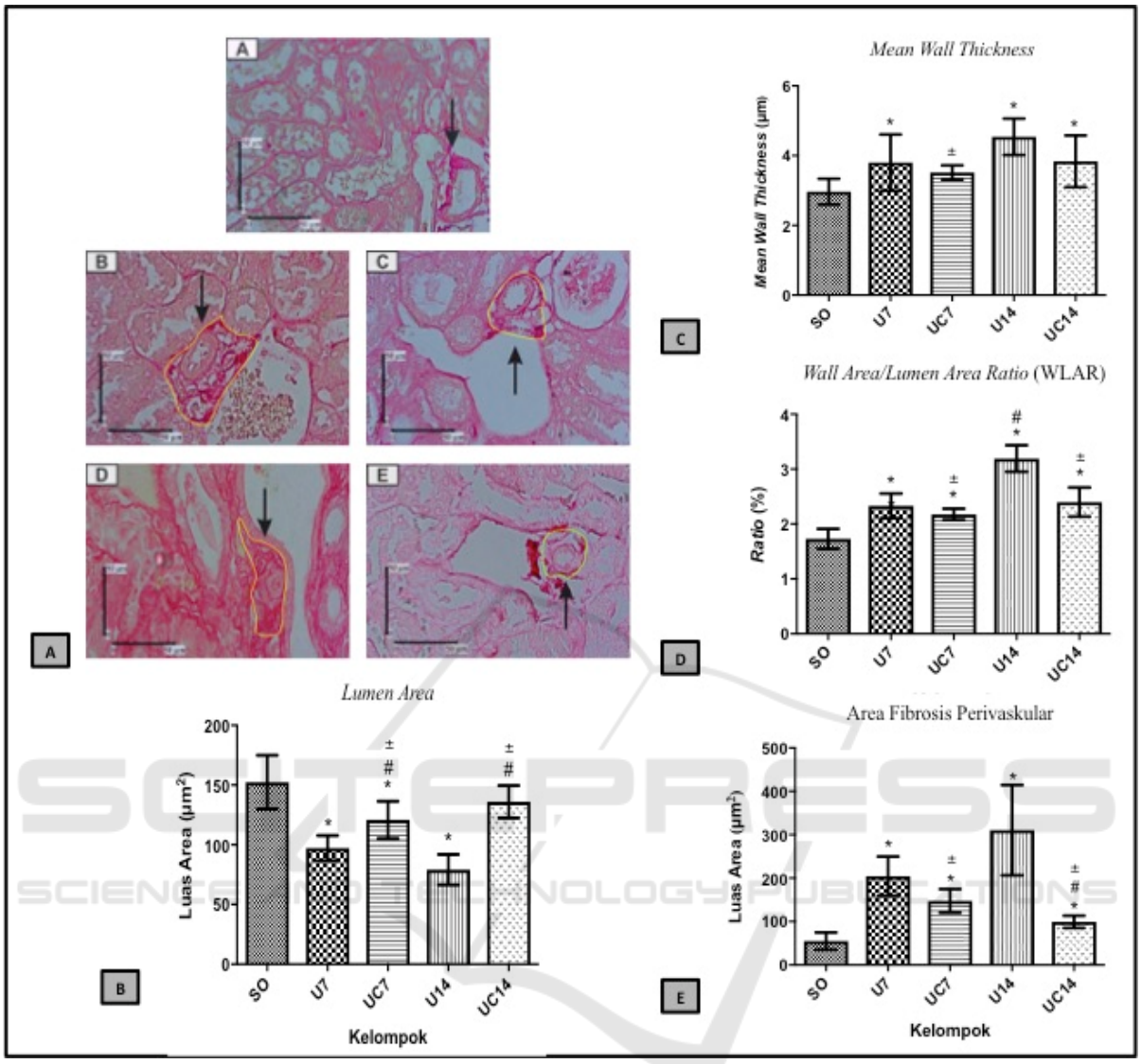


Figure 1. A. Histopatologic view of vessels with vascular remodelling and perivascular fibrosis, A: SO group, B: U7 group, C: UC7 group, D: U14 group, E: UC14 group. B-E. Results of quantitative analysis of lumen area, mean wall thickness, WLAR, and perivascular fibrosis. *=p<0.05 vs SO; #=p<0.05 vs U7; ±=p<0.05 vs U14.

## 4 DISCUSSION

Unilateral Ureteral Obstruction (UUO) is an experimental kidney injury model that provides a representative description of pathological conditions in CKD because it can trigger kidney fibrosis characterized by apoptosis, interstitial fibrosis, glomerulosclerosis, and decrease of kidney mass (Chevalier *et al,* 2009). There was an increase of ROS in UUO due to decreased potential antioxidants, which activated free radicals bioavailability so that oxidative stress occurred (Modaresi *et al,* 2015). Oxidative stress, initiated due to an increase in ROS, was a trigger factor for endothelial dysfunction, marked by a decrease in NO level, vascular remodeling, and cellular damage (Craige *et al,* 2015). Vascular remodeling is a complex process involving endothelial cells, smooth muscle cells, and fibroblast cells. Vascular remodeling represents structural changes like hypertrophy or hyperplasia of vascular smooth muscle cells and extracellular matrix components, which induce changing the artery's mechanical function. Vascular remodeling can be observed by assessing lumen area changes, mean wall

thickness, and the wall area/lumen area ratio (Tanaka & Laurindo, 2017).

In this study, the UUO group showed vascular remodeling; meanwhile, in CGA group showed the ameliorate of vascular remodeling (Figure 1). There was an increase in ROS and an oxidation-reduction reaction, by which c-Jun N-terminal Kinase (JNK) was made and proliferation and hypertrophy leading to vascular injury and vascular remodeling in UUO-induced kidney fibrosis. Reactive Oxygen Species (ROS) modulated intracellular Ca2+ level as a primary factor of cellular activity (Görlach *et al,* 2015). Chlorogenic acid could inhibit the ROS-modulated Ca2+ influx and restored the viability of cells and endothelial cells. Other than that, chlorogenic acid could suppress oxidative stress, inflammation, apoptosis, and autophagy by enhancing kidney regeneration (Domitrović *et al,* 2014). Chlorogenic acid was known to decrease JNK pathway activation leading to inhibition of apoptosis, contraction, migration, and inflammation and reduction of oxidative damage induced by H2O2 (Yu *et al,* 2016). We observed increasing vascular remodeling with lower lumen area, higher mean wall thickness, and WLAR in UUO might associate with vasoconstrictor and vasodilator balance might play a role in regulating vascular remodeling in UUO. Nitric oxide (NO) plays an essential role in regulating vessel tonus and remodeling (Farris & Colvin, 2012). Kidney vasculature also has a high sensitivity to NO. NO released in the medulla induces local blood flow and improves RBF in CKD model (Savard *et al,* 2012).

Besides affecting vascular remodeling, chlorogenic acid also played roles in perivascular fibrosis. We observed higher fibrosis perivascular area in the UUO group. Meanwhile, CGA might ameliorate fibrosis perivascular as shown by lower fibrosis perivascular area in the CGA treated UUO group (Figure 1). Perivascular fibrosis played active roles in developing kidney fibrosis, which was mediated by TGF-B induced myofibroblast transformation (Kramann & Humphreys, 2014). Increased ROS caused an imbalance between oxidation-reduction and modulated the production of TGF-B through *Smad pathway* (Liu & Desai, 2015). Chlorogenic acid had anti-oxidative effects by decreasing TGF-B gene expression and cytokines responsible for fibrosis development through miR-21, which regulated the Smad 7/TGF-B pathway. It showed that chlorogenic acid was an antifibrosis agents (Yang *et al,* 2017). As a result, CGA treatment ameliorated vascular remodeling; also reduced perivascular fibrosis in kidney fibrosis.

# 5 CONCLUSION

In conclusion, that study highlighted the effect of chlorogenic acid ameliorated vascular remodeling based on wider lumen area, thinner mean wall thickness and lower WLAR; ameliorated perivascular fibrosis based on the lower area. For a further research, it is necessary to measure vascular remodeling using *vessel myograph*, which could evaluate endothelial function due to vasoconstriction and vasodilatation.

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