ORIGINAL ARTICLE

Ethanolic Extract of *Centella asiatica* Ameliorates Kidney Ischemia/reperfusion Injury Through Inhibition of Inflammatory Process

Nur Arfian[1], Arindira Maharani[2], Elida Fadhilatul Latifa[2], Indhah Kusumaningtyas[2], Monica Agnes Witono[2], Kemala Athollah[2], Ratih Kemalasari[2], Gita Mumtarin Dara[2], Wiwit Ananda Wahyu Setyaningsih[1], Dwi Cahyani Ratna Sari[1]

[1] Department of Anatomy, Faculty of Medicine, Public Health, and Nurisng, Universitas Gadjah Mada, Yogyakarta, Indonesia.
[2] Undergraduate Program of Medical Doctor, Faculty of Medicine, Public Health, and Nurisng, Universitas Gadjah Mada, Yogyakarta, Indonesia.

ABSTRACT

Introduction: Kidney ischemic-reperfusion injury (IRI) is the main cause of acute kidney injury (AKI) which leads to the inflammation epithelial apoptosis and interstitial fibrosis as the chronic consequenses. *Centella asiatica* (*CeA*) has been known to have various pharmacological effects such as, anti-inflammatory, antioxidant, anti-fibrosis, and, anti-apoptosis. We aimed to elucidate the role of *CeA* in inhibiting kidney injury and infammatory mediators due to kidney IRI. **Methods:** Kidney IRI were performed with bilateral renal pedicles clamping in Swiss background mice (3 months-old, 30-40 grams) for 30 minutes (IR group, n=6), then terminated at day 7 after operation. At the next day, the mice that have been underwent bilateral kidney IRI were administered per-orally with ethanolic extract of *CeA* (210 mg/kg of BW, CeA1 group, n=6, and 420 mg/kg of BW, CeA2 group, n=6). The Sham Operation (SO group, n=6) was used as control. At the day 7 after the surgery, the mice were sacrificed and the kidneys were harvested. The kidney was used to assess tubular injury, interstitial fibrosis, and macrophage number, and another kidney was used to assess the mRNA expression of TLR4. Data were quantified using SPSS 22. **Results:** Kidney IRI produced significantly higher tubular injury, interstitial fibrosis and macrophage number ($p<0.05$) compared to SO with upregulating TLR4 mRNA expression ($p<0.05$). *CeA* treatment attenuated the tubular injury, interstitial fibrosis, macrophage number, and TLR4 mRNA expression which obviously shown in higher-dose of *CeA* ($p<0.05$). **Conclusion:** *CeA* ameliorates tubular injury, kidney fibrosis, and inflammatory mediators due to kidney IRI.

Keywords: *Centella asiatica*, Kidney ischemic-reperfusion injury, Tubular injury, Interstitial fibrosis, Inflammation

Corresponding Author:
Nur Arfian, PhD
Email: nur_arfian@ugm.ac.id
Tel: +62-2746492521 / +62-8112640306

INTRODUCTION

Acute Kidney Injury (AKI) is strongly associated with increased both long term and short-term morbidity and mortality rate. AKI can increase the risk of Chronic Kidney Disease (CKD) and End Stage Renal Disease (ESRD) (1). The progression of AKI to CKD and ESRD could occur faster in people with history of CKD (2). Kidney ischemic/reperfusion injury (IRI) is a common cause of AKI. Kidney transplantation has a high risk of ischemic/reperfusion injury (3). Acute kidney injury in developing countries often occurs due to ischemic/reperfusion injury, as a result of volume depletion due to severe diarrhea, vomiting, and/or shock (4). Several studies of AKI in experimental animals have used a model of kidney ischemic/reperfusion injury (IRI) (5).

Kidney ischemic-reperfusion injury (IRI) causes an imbalance between oxygen supply and demand which leads to the accumulation of metabolic waste that causes tubular epithelial cell death (6). Tubular damage and other mechanisms that occur due to hypoxic conditions cause impairment of kidney function (7). The pathophysiology of kidney IRI is still not fully known, but several mechanisms that contribute to ischemia/reperfusion injury have been formulated (8). Malek and Nematbaksh mentioned that one of the mechanisms involved in ischemia/reperfusion injury is inflammation. Inflammation will aggravate the initial damage to the kidneys that has occurred due to renal ischemic conditions (9).

Many components are involved in the inflammatory response. One of the main components that play an important role in the inflammatory response is macrophages (6). The number of macrophages is increased after ischemic/reperfusion injury. Macrophages play a role in producing Reactive Oxygen Species

(ROS) and various inflammatory mediators which then aggravate ischemic reperfusion injury (10). In addition to macrophages, the toll-like receptor 4 (TLR4) is also known to have an important role in the inflammatory cascade of renal ischemic/reperfusion injury. Toll-like receptors are pattern recognition receptors to detect pathogens and material released by the damaged cells. TLR4 will detect material released from damaged cells during ischemic/reperfusion injury, and trigger activation of the innate immune response which has an important contribution in the inflammatory cascade of ischemic/reperfusion injury. The study revealed that TLR4 inhibition has a protective effect on kidney ischemic/reperfusion injury (11).

The treatment with herbal plants is widely developed and is believed to be an alternative therapy for various diseases. There have been many studies on herbal substances that are beneficial in kidney healing, one of which is *Centella asiatica* (*CeA*). This plant is a tropical medicinal plant that thrives in Southeast Asian countries, including Indonesia (12). *CeA* has been widely consumed both as spices, vegetables, and has been processed into drinks since centuries ago. In Malaysia and Indonesia, this plant is known as the gotu kola (13). The main components of *Centella asiatica* are saponin (glycoside), namely asiatic acid, madecasic acid, terminolic acid, and triterpenes glycoside ester derivatives (asiaticosides, A asiaticoside, B asiaticoside, and madecassoside) (14).

CeA belongs to the Apiaceae family. It extract has been shown to have extensive pharmacological roles, such as wound healing, scar management, and anticancer activity (12). Anti-inflammatory effect of ethanolic extract of *CeA* believes inhibit inflammatory process in kidney disease (15). The asiatic acid component has been known to have various pharmacological effects such as, anti-inflammatory, antioxidant, anti-fibrosis (16), anti-apoptosis, and anti-glycative (17). Madecassoside is also known to have anti-inflammatory and antioxidant activity (12). Asiaticoside has been shown to be effective in enhancing immune responses and has a role in anti-inflammatory, anti-viral, and accelerating wound healing (18). Triterpenes in *CeA* have also been shown to reduce ischemic/reperfusion-injury in some organs in animals, including myocardial infarction and cerebral ischemic reperfusion (19). This study elucidated effect of *CeA* extract in kidney IRI, especially in TLR-4 expression.

MATERIALS AND METHODS

Kidney Ischemic/Reperfusion injury (IRI) model

Kidney ischemic-reperfusion injury (IRI) was performed with bilateral renal pedicles clamping for 30 minutes in twenty-four male Swiss background mice (3 months old, 30-40 grams) which were obtained from the Experimental Animal Care Unit (UPHP) LPPT of Universitas Gadjah Mada. Mice were randomly divided into 4 groups: Sham operated mice for control (SO group, n=6), mice with kidney IRI model (IR group, n=6), mice with IR and treated with 2 doses of ethanolic extract of *CeA*, which were 210 (CeA1 group, n=6) and 420 (CeA2 group, n=6) mg/Kg body weight. Kidney IR was performed to induce acute kidney injury (AKI). Briefly, mice were anesthetized with sodium pentobarbital (10 mg/kg) intraperitoneally. The abdomen was opened, renal pedicles were visualized then clamped with non-traumatic vascular clamp for 30 minutes, then reperfused. Mice were terminated at day7 after kidney IRI procedure.

Mice were housed in a 50x30x15 cm plastic cage according to their group with maximum 3 mice each cage. The cage environment was kept under 12:12 hours natural light-dark cycle, 21°C temperature, and humidity of 40-60%. Mice were fed with standard chow and water ad libitum. This study was done after approval from Ethical Committee of Faculty of Medicine, Universitas Gadjah Mada, based on a statement letter of ethical expediency (KE/FK/0190/EC/2017).

Centella asiatica ethanolic extract

The *Centella asiatica* (*CeA*) leaves were obtained from Merapi Farma Herbal, Kaliurang, Sleman, Special Region of Yogyakarta. Then, it was macerated with 70% ethanol (the absolute ethanol was dissolved in distilled water) in the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada. The ethanolic extract of *CeA* was administered using oral gavage single dose after the surgery for 7 days. Each mouse received 0.3 mL of ethanolic extract of *CeA*. The *CeA* dose was chosen according to our previous study (20).

Kidney harvesting

Mice were terminated at day 7 after operation. Mice were anesthetized with sodium pentobarbital (10 mg/kg) intraperitoneally and then the abdomen and thorax were opened to visualize the heart and kidney. Organs were perfused with 0.9% NaCl from left ventricle using Perista pump (Atto®; Cat. No. SJ-1211H). The left kidney was harvested, half was kept in RNA later® for RNA extraction and the other half was fixated in 4% PFA in PBS for paraffin making.

Interstitial fibrosis area fraction and tubular injury quantification

Paraffin sections with 4µm of thickness was deparaffinized and stained with sirius red (SR) to quantify the fraction area of interstitial fibrosis. The image was captured using the OptiLab software (Olympus; Cat. No. CX22) in 400x magnification of 15 fields each sample. The areas were randomly chosen in cortex and medulla. The interstitial fibrosis area fraction was qualitatively quantified using ImageJ software.

The tubular injury scores were determined through a semiquantitative scoring system. Ten-fields were examined for each kidney, and the lesions were graded from 0 to 4 (0, no change; 1, changes affecting <25%

of the section; 2, changes affecting 25 to 50% of the section; 3, changes affecting 50 to 75%, and 4, changes affecting more than 75% of the section), according to the area with tubulointerstitial lesions (tubular atrophy, tubular dilatation, loss of brush border intraluminal casts, interstitial inflammation and fibrosis). The score index of each mouse was expressed as a mean value of all scores obtained.

Immunohistochemistry (IHC) staining of CD68

The paraffin sections were cut into 4µm thickness then deparaffinized and rehydrated. Then, the slides were heated using for antigen retrieval purposes using citrate buffer pH 6 (Biogear, BGCB-002) for 15 minutes. After that, the slides were incubated using background snipper (Biocare, BS966lL10) followed by primary antibody of anti-CD68 (1:200 dilution, Abcam, ab955) incubation for overnight. At the next day, the slides were incubated with secondary antibody for 1 hour, and DAB solution with same kit (Biocare, STU700L10). Then, the slides were captured using OptiLab software (Olympus; Cat. No. CX22) in 400x magnification of 20 fields each sample.

Reverse Transcriptase-PCR (RT-PCR)

The RNA from kidney tissue was extracted using Trizol RNA solution (GENEzolTM; GZR100) and the RNA concentration was quantified using nanodrop. The cDNA was synthesized using 5xRT-*buffer* (Toyobo, TRT-101), random primer (TAKARA®, 3801), dNTP (TAKARA®, 4030), ReverTra-Ace (Toyobo®; TRT-101). Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to examine the following cDNAs: TLR4 (F: 5′-GGGCCTAAACCCAGTCTGTTTG-3′, R: 5′-GCCCGGTAAGGTCCATGCTA-3′); and GAPDH (F: 5′-TGTGTCCGTCGTGGATCTGA-3′, R: 5′-TTGCTGTTGAAGTCGCAGGAG-3′). PCR conditions were 94°C denaturation for 10 s, annealing at 60°C for 30 sec and extension 72°C for 1 min final extension phase ends with the conditions of 72°C for 10 minutes.

Statistical analysis

Data were analyzed by using one-way ANOVA test for normally distributed and Kruskal-Wallis for data were not normally distributed. The values of $p<0.05$ were considered statistically significant. Statistical analyses were accomplished using SPSS Software version 22.0 (SPSS Inc., Chicago).

RESULTS

***CeA* ameliorated tubular injury and interstitial fibrosis in kidney IRI**

Kidney ischemic-reperfusion injury (IRI) markedly enhanced an increase of tubular injury and interstitial fibrosis. We showed day 7 of IR induced a significant increase of tubular injury ($p<0.01$, $p=0.008$) and interstitial fibrosis ($p<0.01$, $p=0.008$). After *CeA* treatment, the tubular injury ($p<0.01$, $p=009$) and interstitial injury ($p<0.01$, $p=0.014$) were lower significantly, however, low-dose of ethanolic extract of *CeA* more obviously ameliorated tubular injury ($p<0.01$, $p=016$) and interstitial fibrosis ($p<0.01$, $p=0.021$) (Figure 1).

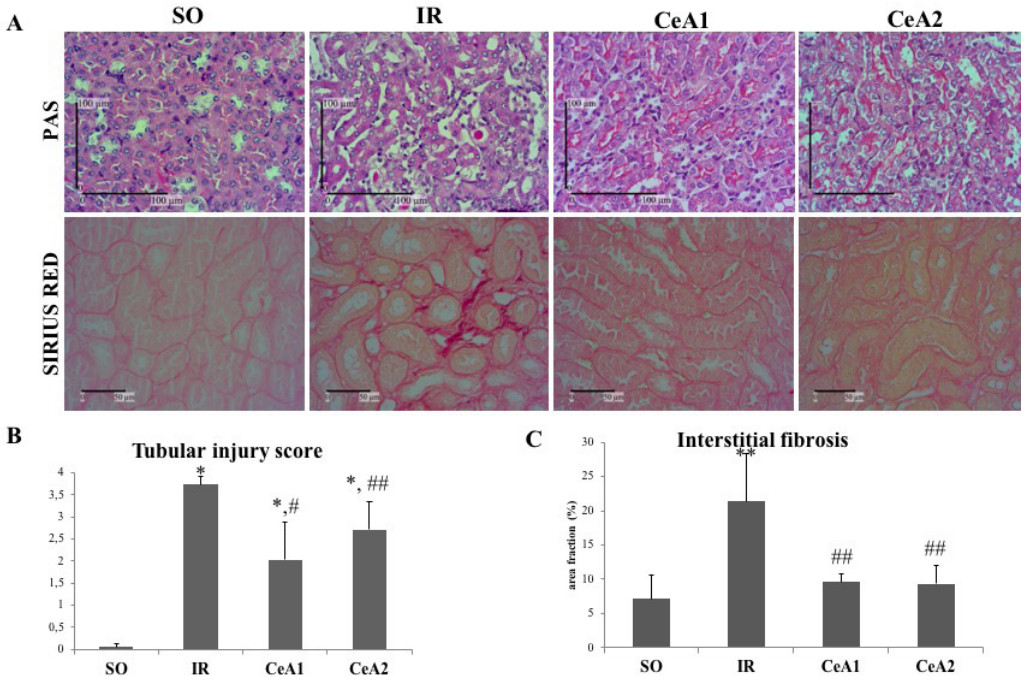

Figure 1: *Centella asiatica* reduced tubular injury and interstitial fibrosis. A. Representative figures of tubular injury and interstitial fibrosis. B-C. Effect of ethanolic extract of CeA in kidney ischemic/renal injury. The tubular injury was highly profound in IRI group and decreased after CeA treatment. The percentage of interstitial fibrosis was remarkably expressed IRI group, then it ameliorated after CeA treatment. Scale bar 100µm. Objective 400X. The data represent average±SD. *=$p<0.01$ vs SO, **=$p<0.05$ vs SO, #=$p<0.01$ vs IR, ##=$p<0.05$ vs IR.

CeA inhibited upregulation of TLR4 and CD68 mRNA expression in kidney IR injury

Then, we showed that kidney IR remarkably increased the CD68 protein expression ($p<0.01$, $p=0.000$) based on IHC quantification and TLR4 mRNA expression ($p<0.05$, $p=0.016$) compared to the SO group. Administration of high-dose of *CeA* (CeA2 group) afforded downregulation of the CD68 protein expression ($p<0.01$, $p=0.000$) and TLR4 mRNA expression ($p<0.05$, $p=0.014$). Low-dose *CeA* (CeA1 group) more remarkably inhibited the macrophage accumulation with lower macrophage number, however there was no significant difference of TLR4 mRNA expression compared to IR group (Figure 2).

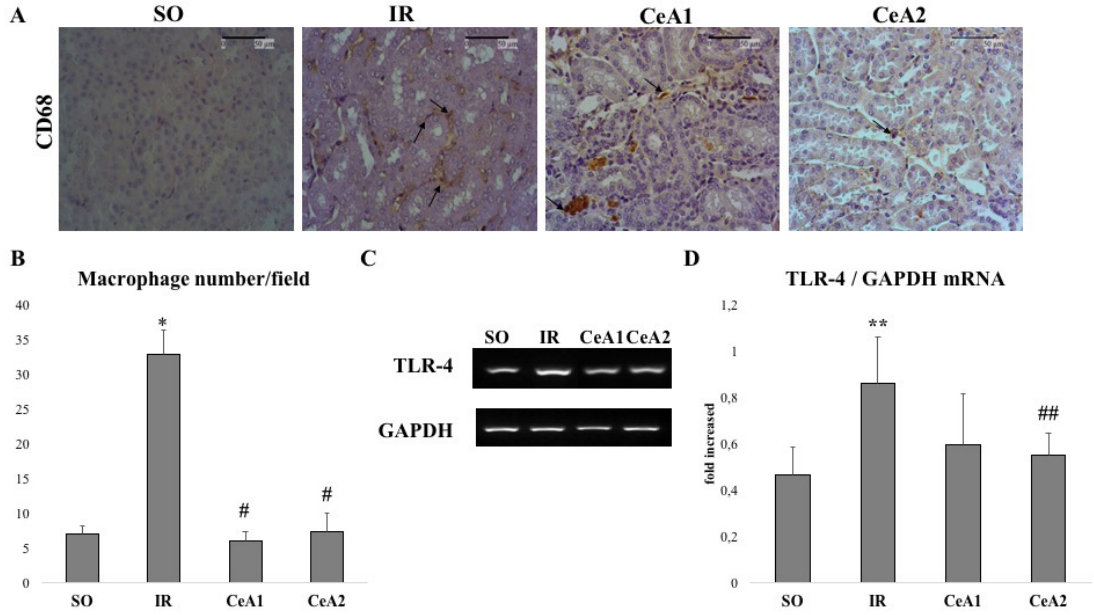

Figure 2: Inflammatory mediators were downregulated during *CeA* treatment. A-B. Representative image and quantification of total macrophage number in IR group. After *CeA* treatment, the TLR4 mRNA expression and CD68 protein expression downregulated. Scale bar 100μm. Objective 400X. The data represent average±SD. *=$p<0.01$ vs SO, **=$p<0.05$ vs SO, #=$p<0.01$ vs IR, ##=$p<0.05$ vs IR.

DISCUSSION

Kidney IRI causes an imbalance between the oxygen supply and demand, and the accumulation of metabolic waste that causes tubular epithelial cells to damage, and then to death (6). The degree of tubular injury is assessed by the loss of brush borders, epithelial thinning/atrophy, epithelial dilation, and presence of intraluminal cast. We showed that the IR group had the highest tubular injury compared to the SO group. The severity of tubular injury in the IR group was clearly evident with the loss of brush borders in the apical proximal tubular epithelial cells. During the ischemic period, the cytoskeleton is distorted, causing missed location of adhesion molecules (tight junction and adherent junction) and disturbing the polarity of proteins in basolateral and apical epithelial cells. These events cause the brush borders of microvilli dislodged into the lumen (3). The tubular epithelial cells undergo thinning or atrophy due to the detachment of the apoptotic tubular cells from the basement membrane. The living tubular cells are also detached from the basement membrane, and migrate to the basement membrane left by the apoptotic cells and then differentiate. Epithelial thinning causes leakage from the lumen to the peritubular interstitial, especially in the increased intraluminal pressure due to cellular debris so that the tubular lumen appears dilated (21). The IR group had more intraluminal cast than the other groups. Intraluminal cast is formed by a high Na+ concentration so the Tamm-Horsfall monomeric glycoprotein is converted into a gel-like polymer, then joins the necrotic tubular cells and microvilli brush borders (22).

In our study, the group treated with *CeA* ethanolic extract (CeA1 and CeA2) had lower severity of tubular injury than the IR group, although higher than the SO group. Improvement of IRI is indicated by attenuation of the tubular injury severity (23). According to Devajaran (2006), the initiation phase (loss of brush border and damage to the polarity and cytoskeleton of cells) in ischemic/reperfusion injury will continue to the extension phase (cell apoptosis, desquamation, lumen obstruction, and inflammatory response), then the maintenance phase will occur when there is a balance between tubular cell death and repair (as a result of the differentiation of living tubular cells). The administration of *CeA* can be expected to accelerate the progression from the extensive phase to the maintenance phase and prevent the progression from the initiation phase to the extension phase. It significantly reduced the severity of tubular injury in the kidney IRI in mice. Unfortunately, there have been no studies on the effects of *CeA* extract

on kidney IRI, so the renoprotective pathway of *CeA* is still unknown. Most existing studies include the effects of *CeA* extract on ischemic/reperfusion injury in organs other than the kidneys, such as the heart and cerebrum.

The *CeA* group showed low degree of tubular injury than IR group. It can be explained by the antioxidant effects of *Centella asiatica* extract. ROS plays a role in the pathophysiology of ischemic/reperfusion injury by causing protein oxidation, lipid peroxidation, DNA damage, and inducing cell apoptosis (24). In cerebral ischemic/reperfusion studies, *Centella asiatica* extract increases the work efficiency of superoxide dismutase (SOD) and gluthatione (GSH) as antioxidant (25). Asiatic acid which is an active substance in *Centella asiatica* increases the activity of catalase, SOD, and glutathione peroxidase (GPx) (26). The pathophysiology of kidney IRI also involves ROS. The administration of *CeA* causes a decrease in ROS so that the inducible nitrite oxide synthase (iNOS) expression is inhibited, then decreases NO production through NF-κB (noncanonical pathway) inactivation (27). The reduction of ROS reduces the disruption of the cytoskeleton in tubular cells, and maintains the integrity and viability of tubular cells. Thus, the group treated with *Centella asiatica* showed lower degree of tubular injury than IR group.

A study conducted by Prakash & Kumar (2012) using a mice model of neurotoxicity induced by aluminium revealed that the active component of *CeA* causes the reduction of Caspase activity thereby reduces the number of apoptotic cells. This is similar to the apoptotic mechanism in kidney ischemic/reperfusion injury through the caspase pathway (7,28).

Kidney IRI leads to abnormal repair of the kidneys which induces post-ischemic fibrosis (6). The degree of renal interstitial fibrosis after IRI was measured histologically by calculating the fibrosis fraction area with Sirius red staining. This is in accordance with previous studies which stated an increase in collagen production as a component of the extracellular matrix after kidney IRI (6,29).

The main role in producing extracellular matrix is myofibroblasts. Based on the theory, there are 5 mechanisms regarding the myofibroblast formation: interstitial fibroblast activation, pericyte differentiation, phenotypic conversion of tubular epithelial cells and endothelial cells, and aggregation of circulating fibrocytes. This activation process of interstitial fibroblasts and epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition (EndoMT) is mediated by one of profibrogenic cytokines, TGF-β1 (29).

Our study also showed reduction of macrophage accumulation with downregulating TLR4 mRNA expression in CeA2 group. Inflammation is an important component in the pathogenesis of kidney IRI (8). Toll-like receptor 4 (TLR4) is a component that induces innate immune response that causes inflammation in kidney ischemic/reperfusion injury (30). During acute kidney injury, TLR4 activation initiates an inflammatory response characterized by the production of chemokines, attracting the immune cells, and producing pro-inflammatory cytokines via the NF-κB pathway. TLR4 expression increases after kidney IRI. The study stated that TLR4 expression in tubular epithelial cells increased on the first day to day 5 after reperfusion (31).

Wu, et al. (2010) found out that an increase in TLR4 expression was triggered by an increase in endogenous ligands from TLR4, such as high mobility group box 1 (HMGB1) protein and bigcerans. HMGB1 is an inflammatory mediator in initial phase of kidney IRI (32). HMGBI is a protein found in nucleus of almost all eukaryotic cells. HMGB1 works to stabilize the nucleosomes and acts as a transcription factor. HMGB1 is released passively from damaged/dead cells due to kidney ischemic conditions. The study found that HMGB1 expression rose on the first day to day 5 after reperfusion. Research revealed that inflammatory factors HMGB1 play a role in linking early cell damage with an inflammatory cascade during kidney ischemic reperfusion injury. The study also showed that HMGB1 inhibition may reduce the inflammation in kidney ischemic/reperfusion injury (33). The activation of TLR4 by HMGB1 causes the activation of NF-κB. NF-κB activation further triggers transcription of pro-inflammatory cytokines such as TNF-α, IL-1β, MCP-1, and IL-6. The cytokines then bind to their receptors on renal tubular cells. The bond between the ligand and receptor will cause an inflammatory cascade that worsen kidney damage. Research shows inhibition of the HMGB1-TLR4 pathway can reduce kidney damage due to ischemic/reperfusion injury (32).

The number of macrophages after kidney IRI increased rapidly and peaked on day 7 and remained high on day 14 (34). Another study done by Boventre and Yang (2011) revealed that the number of macrophages immediately rose at 1 hour after IRI, peaked after 24 hours, and lasted up to 7-14 days (6). Our study showed significantly increased number of macrophages in IR compared to SO group. According to the previous studies which stated that the number of macrophages increased 7 days after kidney ischemic/reperfusion injury. Macrophage infiltration into kidney tissue is mediated by C-C motifs of chemokine receptor 2 (CCR2) and C-X3-C motifs of chemokine receptor 1 (CX3CR1), both of which are receptors for different chemokines. Monocyte chemoattractant protein-1 (MCP-1) is the main ligand for CCR2, while CX3CR1 is a receptor for fractalkine ligand. CCR2 and CX3CR1 are known to play an important role in the accumulation of macrophages

to kidney tissue (35).

MCP-1 is the most potent chemoattractant in attracting monocyte. There was a significant increase in MCP-1 two hours after reperfusion. The study showed that the increase in MCP-1 was followed by an increase in the NF-κB transcription factor. Previous research also found that NF-κB inhibition had an effect on the inhibition of mRNA MCP-1. This showed that the increase in MCP-1 is mediated by NF-κB. This increase in MCP-1 plays an important role in increasing macrophage infiltration after kidney ischemic/reperfusion injury (36). *Centella asiatica* has been reported to have anti-inflammatory effects (37). The main active substances in *Centella asiatica* are saponins/triterpenoids, including asiaticosides and madecasoside. Madecassoside (MA), the main terpenoid in *Centella asiatica*, is known to have a protective effect on ischemic/reperfusion injury to the heart through the Akt/GSK-3/HIF-1 pathway (38). *Centella asiatica* has also been shown to reduce the expression of TLR2 and TLR4 in striatum of mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (37). This study was revealed the effect of *Centella asiatica* on attenuation of tubular injury, interstitial fibrosis, and macrophage infiltration in mice with kidney IRI. Furthermore, in higher dose of *CeA* (420 mg/Kg of BW) treatment, we found reduction of macrophage infiltration and TLR4 mRNA expression.

CONCLUSION

In conlusion, the *Centella asiatica* has a potent anti-inflammatory effect which can be used to inhibit macrophage accumulation and TLR4 mRNA expression which leading to inhibition of tubular injury and interstitial fibrosis in kidney IRI model.

ACKNOWLEDGEMENTS

We would like to thank to Mr. Mulyana for animal maintenance. Some of the data in this manuscript were used for finishing the Bachelor Degree in Medical Doctor of Arindira Maharani, Elida Fadhilatul Latifa, and Indhah Kusumaningtyas, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia. This research was funded by Dana Masyarakat Scheme, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada and Rekognisi Tugas Akhir (RTA), Universitas Gajah Mada.

REFERENCES

1. Coca SG, Singanmala S, Parikh CR. Chronic Kidney Disease after Acute Kidney Injury: A Systematic Review and Meta-analysis. Kidney Int. 2013;81(5):442–8.
2. Bonventre J V, Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest. 2011;121(11).
3. Pol P van der. Pathogenic role of complement in renal ischemia/reperfusion injury. 2013.
4. Perico N, Remuzzi G. Acute kidney injury in low-income and middle-income countries : no longer a death sentence. Lancet Glob Heal. 2016;4(4):e216–7.
5. Wei Q, Dong Z. Mouse model of ischemic acute kidney injury : technical notes and tricks. Am J Physiol Ren Physiol. 2012;303:F1487–94.
6. Bonventre J, Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest. 2011;121(11):4210–21.
7. Devarajan P. Update on Mechanisms of Ischemic Acute Kidney Injury. Am Soc Nephrol. 2006;17(1):1503–20.
8. Malek M, Nematbakhsh M. Renal ischemia/reperfusion injury;from pathophysiology to treatment. J Ren Inj Prev. 2015;4(2):20–7.
9. Lu CY, Hartono JR. Acute kidney injury: a conspiracy of toll-like receptor 4 on endothelial, leukocytes, and tubules. Pediatr Nephrol. 2012;27(10):1847–54.
10. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxid redox Signal. 2014;20(7):1126–67.
11. Dagher PC, Hato T, Mang HE, Plotkin Z, Richardson QV, Massad M, et al. Inhibition of Toll-Like Receptor 4 signaling mitigates microvascular loss but not fibrosis in a model of ischemic acute kidney injury. Int J Mol Sci. 2016;17:647.
12. Luo Y, Yang Y, Liu J, Li W, Yang J, Sui X, et al. Neuroprotective effects of madecassoside against focal cerebral ischemia reperfusion injury in rats. Brain Res. 2014;1565:37–47.
13. Pan Y, Abd-rashid BA, Ismail Z, Ismail R, Wah J. In vitro modulatory effects on three major human cytochrome P450 enzymes by multiple active constituents and extracts of *Centella asiatica*. J Ethnopharmacol. 2010;130:275–83.
14. Raghavendra M, Maiti R, Kumar S, Trigunayat A, Mitra S, Acharya SB. Role of *Centella asiatica* on cerebral post-ischemic reperfusion and long-term hypoperfusion in rats. Int J Green Pharm. 2009;(June):88–96.
15. Arfian N, Setyaningsih WAW, Anggorowati N, Romi MM, Sari DCR. Ethanol extract of *Centella asiatica* (Gotu Kola) attenuates tubular injury through inhibition of inflammatory cytokines and enhancement of anti-fibrotic factor in mice with 5/6 subtotal nephrectomy. Malaysian J Med Sci. 2019;26(5):53–63.
16. Meng X, Zhang Y, Huang X-R, Ren G, Li J, Lan HY. Treatment of renal fibrosis by rebalancing TGF-β/Smad signaling with the combination of asiatic acid and naringenin. Oncotarget. 2015;6(35):36984–97.
17. Huang S, Chiu C, Chen H, Hou W, Sheu M, Lin Y, et al. Antinociceptive Activities and the Mechanisms of Anti-Inflammation of Asiatic Acid

in Mice. 2011;2011.
18. Wang Z, Liu J, Sun W. Effects of asiaticoside on levels of podocyte cytoskeletal proteins and renal slit diaphragm proteins in adriamycin-induced rat nephropathy. Life Sci. 2013;93(8):352–8.
19. Ian GB, Ang YY, Iu RL, En JR, En LW, Uo SG. Madecassoside reduces ischemia-reperfusion injury on regional ischemia induced heart infarction in rat. Biol Pharm Bull. 2008;31(3):458–63
20. Sari DCR, Arfian N, Tranggono U, Setyaningsih WAW, Romi MM, Emoto N. Hippocampal brain-derived neurotrophic factor (BDNF), tyrosine kinase B (TrkB) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) signaling in chronic electrical stress model in rats. Iran J Basic Med Sci. 2019;22(8):1218–24.
21. Asada N, Takase M, Nakamura J, Oguchi A, Asada M, Suzuki N, et al. Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. J Clin Invest. 2014;19(1):3981–90
22. Yoshida M, Honma S. Regeneration of injured renal tubules. J Pharmacol Sci. 2014;124(2):117–22.
23. Hafez MS. The effect of pentoxifyllin in a rat model of renal ischemic reperfusion injury , light, transmission, and scanning electron microscopical study. Egypt J Histol. 2012;148–58.
24. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell Biology of ischemia/reperfusion injury Theodore. Int Rev Cell Mol Biol. 2012;298:229–317.
25. Kumari S, Deori M, Elancheran R, Kotoky J, Devi R. In vitro and in vivo antioxidant , anti-hyperlipidemic properties and chemical characterization of *Centella asiatica* (L.) extract. Front Pharmacol. 2016;7(October):1–12.
26. Huang X, Zuo L, Lv Y, Chen C, Yang Y, Xin H, et al. Asiatic acid attenuates myocardial ischemia/reperfusion injury via Akt/GSK-3β/HIF-1α signaling in rat H9c2 cardiomyocytes. molecules. 2016;21:1–14.
27. Yang L, Brooks CR, Xiao S, Sabbisetti V, Yeung MY, Hsiao L, et al. KIM-1–mediated phagocytosis reduces acute injury to the kidney. J Clin Invest. 2015;125(4):1620–36.
28. Prakash A, Kumar A. Mitoprotective effect of *Centella asiatica* against aluminum-induced neurotoxicity in rats : possible relevance to its anti-oxidant and anti-apoptosis mechanism. Neurol Sci. 2013;34:1403–9.
29. Liu Y. Cellular and molecular mechanisms of renal fibrosis. Nat Rev Nephrol. 2015;7(12):684–96.
30. Pulskens WP, Teske GJ, Butter LM, Roelofs JJ, Poll T Van Der. Toll-like Receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. PLoS One. 2008;3(10):1–9.
31. Zhao H, Perez JS, Lu K, George AJT, Ma D. Role of Toll-like Receptor-4 in renal graft ischemia-reperfusion injury. Am J Physiol Ren Physiol 306. 2014;306:F801–11.
32. Zhang J, Xia J, Zhang Y, Xiao F, Wang J, Gao H, et al. HMGB1-TLR4 signaling participates in renal ischemia reperfusion injury and could be attenuated by dexamethasone-mediated inhibition of the ERK/NF-κB pathway. Am J Transl Res. 2016;8(10):4054–67.
33. Wu H, Ma J, Wang P, Corpuz TM, Panchapakesan U. HMGB1 Contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol. 2010;21:1878–90.
34. Kim M, Kim SC, Ko YS, Lee HY, Jo S. The Role of M2 macrophages in the progression of chronic kidney disease following acute kidney injury. PLoS One. 2015;1–17.
35. Li L, Huang L, Sung SJ, Vergis AL, Rosin DL, Edward C, et al. The chemokine receptors CCR2 and CX3CR1 mediate monocyte/ macrophage trafficking in kidney ischemia–reperfusion injury. Kidney Int 2008. 2009;74(12):1526–37.
36. Sung FL, Zhu TY, Au-Yeung KKW, Siow YL, Karimin. Enhanced MCP-1 expression during ischemia/reperfusion injury is mediated by oxidative stress and NF-κB. Kidney Int. 2002;62:1160–70.
37. Choi M, Zheng H, Kim J, Lee K, Park Y, Lee D. Protective effects of *Centella asiatica* leaf extract on dimethylnitrosamine-induced liver injury in rats. Mol Med Rep. 2016;14:4521–8.
38. Huang ALAL, Vita JAJA. Effects of systemic inflammation on endothelium-dependent vasodilation. Trends Cardiovasc Med. 2006;16(1):15–20.