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Chlorogenic Acid Attenuates Inflammation with Upregulating Nrf2 and Downregulating NFκB and CD68 mRNA Expression in 5/6 Subtotal Nephrectomy Model in Mice

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Abstract: Chronic kidney disease is characterized by persistent inflammation. Under ideal conditions, a balance is required between pro-inflammatory and anti-inflammatory factors. Chlorogenic acid (CGA) has anti-inflammatory activity. Twenty-five male Swiss-Webster mice (3-4 months) were divided into five groups; SO (sham operation, n=5), SN for fourteen days (SN14, n=5), SN for twenty-eight days (SN28, n=5), SN with ip CGA injection on day 15-28 (C14, n=5) and SN with ip CGA injection on day 1-28 (C28, n=5). CGA was injected with 14 mg/kgBW. Mice were treated for 14 days (SN14) and 28 days (SN28, C14, and C28) and then euthanized. Next, the kidneys were harvested. mRNA expression of NFκB was identified by qRT-PCR, while mRNA expressions of Nrf2, CD68, and Arg1 were by RT-PCR. The immunohistochemical analysis determined the localization of CD68 and Arg1. CGA administration induced significantly lower mRNA expressions of NFκB in C28 and C14 compared to SN28. Meanwhile, Nrf2 mRNA expression in C28 and C14 was significantly higher than SN28. CD68 immunostaining showed accumulation of macrophages in SN14 and SN28 groups, meanwhile reduced attenuated in C14 and C28 groups, with no change in Arg1 immunostaining. Early administration of chlorogenic acid suppressed inflammation by downregulating NFκB and CD68 expression and upregulating Nrf2 mRNA expression.

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Keywords: chlorogenic acid; subtotal nephrectomy; chronic kidney disease, inflammation; NFκB; Nrf2; CD68 and Arg1.

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1. Introduction

Kidney disease significantly contributes to the global health burden because it causes worldwide morbidity and mortality [1]. An analysis showed that in 2017 the global prevalence of chronic kidney disease (CKD) reached 9.1% (697.5 million cases) and resulted in 1.2 million deaths, ranking it the 12th leading cause of death worldwide [2]. Furthermore, the burden of CKD is estimated to worsen in the upcoming decades [3,4]. CKD can cause end-stage renal

disease with markers such as glomerulosclerosis, tubular injury, anemia, inflammation, and interstitial fibrosis [5]. Fibrosis development is divided into the priming phase, activation phase, execution phase, and progression phase [6]. The priming phase is an inflammatory response that recruits lymphocytes, monocytes/macrophages, dendritic cells, and mast cells [6]. The priming phase is induced by injury to the kidneys. Kidney injury causes the continuous release of Damage-Associated Molecular Patterns (DAMPs) that lead to macrophage infiltration and the release of pro-inflammatory cytokines [7].

Immune cell infiltration in wound tissue will contribute to tissue repair and intrinsic homeostatic restoration, such as macrophage expression. Macrophages can change the basal state as an activation process called macrophage polarization as a response to stimuli [8]. Macrophage polarization in the M1 (pro-inflammatory) is characterized by biomarkers such as CD68 and various Interleukins (IL) such as IL-6 that is mediated by transcription factor Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NFκB) [9] and M2 (anti-inflammatory). M2 is characterized by Arginase-1 (Arg1), IL-4 and IL-10 [10]. IL-10 production is modulated by heme oxygenase (HO) through the activation of Nuclear factor erythroid 2-related factor2 (Nrf2) [11]. HO is involved in regulating macrophage polarization [12] Also, modulating antioxidants enzymes such as HO as antioxidants, and coping with oxidative stress [13]. Under ideal conditions, a balance is needed between pro-inflammatory and anti-inflammatory factors and efforts to repair kidney damage [14].

Accordingly, many studies were developed to produce kidney homeostasis, for example, by targeting inflammation [15]. Not only inflammation but also oxidative stress is very related to CKD [16]. One of the ingredients which have the potential to target inflammation is Chlorogenic Acid (CGA), which can reduce Reactive Oxygen Species (ROS) [17] so that it has an antioxidant effect. In addition, CGA has anti-inflammatory activity. It affects macrophage polarization by suppressing the production of IL-1β, a pro-inflammatory cytokine released by M1 [18], so it is very potent in treating kidney damage, including acute kidney injury (AKI).

Efforts to neutralize inflammation in CKD lead to the restoration of renal hemodynamics, maintaining renal microvascularity, and reducing renal fibrosis [19]. In addition, timely recognition and early treatment of CKD can slow progression while preventing complications and reducing cardiovascular risk [19]. Various studies have examined the effect of giving CGA for the prevention and treatment of CKD, such as CGA's nephroprotective effect against sodium arsenite-induced oxidative stress, inflammation, and apoptosis [20], indicating CGA relieves renal inflammation [21] and can inhibit oxidative stress and inflammation that prevents diabetic nephropathy [22]. However, research that focused on the timing of administration for its anti-inflammatory activities is still limited, whereas the administration requires the right timing since administration at the wrong time can result in severe kidney damage in the progression of CKD. This study was conducted to determine the effect of CGA administration on the progression of chronic kidney failure through the expression of NFκB, Nrf2, and M1 and M2 macrophages when the timing of administration was differentiated on the 14th and 28th days after the occurrence of the chronic renal failure model.

2. Materials and Methods

2.1. Ethical clearance.

This research obtained permission from the Medical and Health Research Ethics Committee (MHREC) Universitas Gadjah Mada, Yogyakarta, based on the Certificate of Ethics Approval with the number: KE/FK/1301/EC/2021.

2.2. Animal model of 5/6 subtotal nephrectomy.

The subject in this study used male Swiss-Webster mice 3-4 months old weighing 30-40 grams that were obtained from the Laboratory of the Faculty of Pharmacy, Universitas Gadjah Mada (Yogyakarta, Indonesia). The cages were placed in a room; room temperature was a light cycle of 12 hours dark and 12 hours light. Experimental animals were divided into 5 treatment groups with laboratory conditions. Group 1 sham operation (SO) was a control group, namely the group that underwent surgery but not subtotal nephrectomy (SN), group 2 was SN14, group 3 was SN28, group 4 was C14 (28-day subtotal nephrectomy group that was injected with CGA (Sigma Aldrich, C3878) at a dose 14 mg/kgBW/day administered intraperitoneally on days 15-28), and group 5, namely C28 (28-day nephrectomy subtotal group that was injected with CGA on days 1-28).

2.3. RNA extraction and cDNA synthesis.

After euthanasia, the kidneys were extracted and cut into small pieces, then stored in a 1.5 ml microtube containing 0.5 ml of general solution and later mashed using a micropestle. After this, chloroform was added to the delicate tissue, homogenized using a vortex, and centrifuged at 4˚C at 13,000 RPM for 15 minutes. The supernatant was formed, then added with isopropanol, then homogenized with a vortex. Centrifugation was carried out again at 13,000 RPM at 4˚C for 15 minutes. The resulting supernatant was discarded, leaving a pellet. 1 ml of 70% ethanol was added to wash the pellet, then centrifuged at 13,000 RPM at 4˚C for 5 minutes. The supernatant was discarded, and the shell formed was dissolved with 50 µl DEPC, then incubated at 55˚C for 10 minutes, and a total RNA solution was produced.

The initial stage of cDNA synthesis was initiated by making a mixture A consisting of total RNA, oligo (dT) 1 µl, and DEPC-Treated H2O 10 µl. Mixture A was then incubated at 70˚C for 5 minutes. Next, mixture B consisted of 4µ l of RT buffer (DTT/dNTPS), 5 µl of DEPC-Treated H2O, and 1 µl of RTase/RI Enzyme Mix. Mixtures A and B were then combined and incubated at 25˚C for 10 minutes, 37-50˚C for 50 minutes, and terminated at 85˚C for 5 minutes. The cDNA results were then stored at -20˚C.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR) and electrophoresis.

Reverse Transcriptase PCR was performed for examination of genes with specific primers: Nrf2 (forward: 5’-GCAGGCTATCTCCTAGTTCTCC-3’; reverse primary: 5’-GCTACTTGCAGCAGAGGTGA-3’. CD68: forward primary: 5’-CATCAGAGCCCGAGTACAGTCTACC-3’; reverse primary: 5’-AATTCTGCGCCATGAATGTCC-3’. Arg1: forward primer: 5’-AACACTCCCCTGACAACCAG; reverse primer: 5’- CCAGCAGGTAGCTGAAGGTC-3’. GAPDH: forward primer: 5’-TGTGTCCGTCGTGGATCTGA-3’: reverse primer: 5‘-

TTGCTGTTGAAGTCGCAGGAG-3'. We used Taq Master Mix (GoTaq®Green Master Mix, Cat No. M7122).

Electrophoresis was started with the manufacture of 2% agarose gel with DNA ladder (Bioron, Germany, Cat No. 306009). Gel photos were performed using a G:BOX Chemi XRQ® doc gel tool with ultraviolet light transillumination.

2.5. qRT-PCR.

qRT-PCR was performed for examination of genes with specific primers NFκB forward primer: 5'- GCGTACACATTCTGGGGAGT-3'; reverse primer: 5'- ACCGAAGCAGGAGCTATCAA-3'. qRT-PCR was performed following the kit PCR protocol (Toyobo THUNDERBIRD SYBR qPCR Mix 2004) consisting of: Thunderbird (qPCR Mix Toyobo, QPS-201), ROX, and NFW by adjusting the thermal cycle of the PCR machine.

2.6. Immunohistochemical (IHC) staining.

Paraffin slides underwent deparaffinization and rehydration. Antigen retrieval was performed using a citrate buffer. Peroxidase inhibition was performed with 3% H_2O_2 for 5 minutes to inhibit endogenous peroxidase and washed with phosphate-buffered saline (PBS) (3x5 minutes). Blocking serum Fine Test dripped onto a slide and covered with parafilm for 20 minutes, then incubated with anti-CD68 antibody (1:100, ab 955), anti-Arginase-I antibody (1: 100, Santa Cruz Biotechnology) and covered with parafilm overnight at 4˚C. On the second day, the preparations were washed with PBS (3x5 minutes), followed by incubation with secondary antibodies, namely poly HRP-Goat Anti-Rabbit for Arg1 and poly HRP-Goat Anti-Mouse CD68; then the slides were covered with parafilm for 60-90 minutes. Before being applied with DAB 1:100 and viewed with a microscope, the slide has washed with PBS (3x5 minutes) and counterstained with hematoxylin. The staining results were observed using a light microscope connected to a 400X magnification computer. IHC was used to examine CD68 and Arg1 localization.

3. Results and Discussion

3.1. Chlorogenic acid (CGA) attenuated inflammation.

RT-PCR test revealed that the subtotal nephrectomy model induced injury in kidney tissue with macrophage infiltration. We found expression of M1 (CD68) macrophages in SN14 and SN28 significantly higher than the SO group, indicated by the macrophage's infiltration [23]. Early injury and inflammation that happens during chronic conditions will increase M1 macrophages, which is around the injury that follows with recruiting of neutrophils, Natural Killer (NK) cells, and Th1/17 [24]. Meanwhile, there was significantly lower CD68 in the C28 group compared to SN14 and SN28. The C28 group was significantly lower compared to C14 (Figure 1C). Early treatment using CGA might be associated with the reduction of inflammation. Localization macrophages using IHC staining showed positive staining of CD68 in the interstitial area. CD68 is expressed in normal tissue but increases when it is stimulated by an inflammatory response [25], so the expression of CD68 is categorized as an M1 marker [26]. Quantifying Arg1 as a biomarker of M2 shows SN14 and SN28 groups tend to be higher than SO. Arg1 mRNA expression tends to be lower in C14 and C28 groups compared to SN14

and SN28 (Figure 1C). The injury that was induced by ischemia-reperfusion caused cell apoptosis in the tubular, which will induce the high expression of CSF/Csf2, which directs to the selective expression of Arg1 in macrophages [27].

The C14 and C28 groups tended to be lower than SN14 and SN28 groups (Figure 1C). M2 is divided into several types that reduce the infiltration of macrophages [28,29]. Arg1, as M2a macrophages, inhibits pro-inflammatory cytokines and produces some anti-inflammatory IL-10 [29]. During inflammation, Th2 and Treg will follow recruiting neutrophils, NK cells, and Th1/17 in the tissues for local immune response, which will cause the number of M2 to be contiguous or none due to inflammation that happens continuously [24]. Some research found that the time of M2 response is crucial to determine which tissue can regenerate into a homeostatic or fibrotic condition [30]. Switching of M1 and M2 phenotypes that were identified in the UUO model revealed that in the early model (by day 3), macrophages polarized into the M1 phenotype and switched into the M2 phenotype (by day 5) [31,32]. Localization of Arg1 by IHC showed that it was expressed in the interstitial areas of the kidneys (Figure 1A).

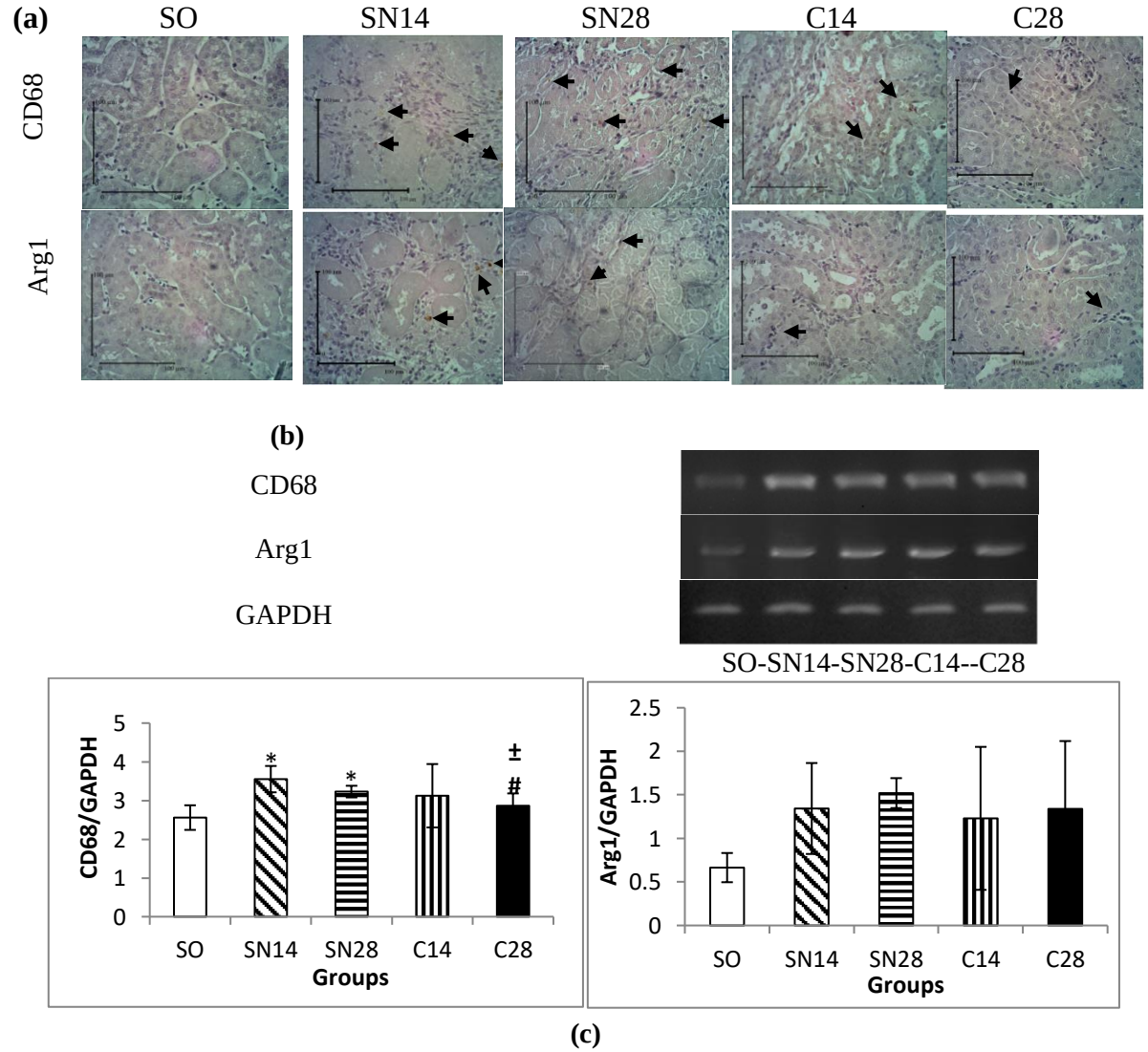
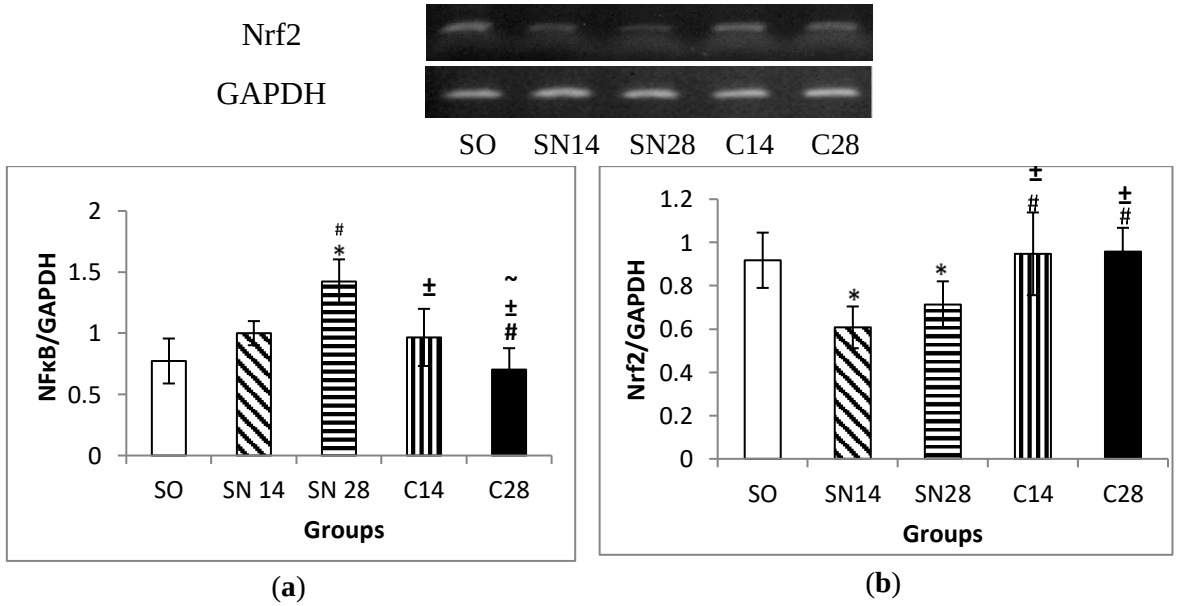


Figure 1. Chlorogenic acid lowered pro-inflammatory (M1) macrophages. (a) immunohistochemistry results showed that subtotal nephrectomy induces infiltration of macrophages, either M1 or M2. (b,c) RT-PCR method was performed for CD68 (M1) and Arg1 (M2). Chlorogenic acid can lower CD68 as a marker of inflammation in C28. *$p < 0.05$ vs SO, #$p < 0.05$ vs SN14, ±$p < 0.05$ vs SN28, ~$p < 0.05$ vs C14.

3.2. Chlorogenic acid (CGA) lowered NFκB and induced higher Nrf2.

qRT-PCR showed that subtotal nephrectomy induces higher production of NFκB as a transcription factor of inflammation [33]. SN14 tended to be higher than SO. It might be associated with the development of progression of CKD if compared to the SO group. The SN28 group was a significantly higher NFκB level than SO (Figure 2 a) [34]. The research revealed that increased p-NFκB protein 28 days in the subtotal nephrectomy model indicated NFκB and p38 MAPK signaling pathways are activated during inflammation [14]. The C28 group was revealed to be significantly lower than the SN14 and SN28 groups (Figure 2a). A previous study showed in a kidney ischemia-reperfusion model that NFκB levels were downregulated after CGA treatment [35], demonstrating CGA with anti-inflammation activity. The reduction of oxidative stress after treatment with CGA is caused by its ability to donate hydrogen atoms that reduce free radicals and inhibit oxidation reactions [17]. CGA-induced Nrf2, which inhibits activation of NFκB through ubiquitination with degradation IKKβ [36]. Our results revealed early treatment of CGA downregulated NFκB mRNA expression in the C28 group compared with C14 [34]. Long-term administration of CGA can attenuate glomerulosclerosis and tubulointerstitial in kidney models with CKD induced by subtotal nephrectomy [37]. It is associated with its antioxidant effects and TGF-Smad pathway blocking [37]. RT-PCR revealed that Nrf2 was expressed lowest in SN14 and SN28 than SO (Figure 2b). It seems that subtotal nephrectomy models can suppress Nrf2, which is involved in antioxidant and anti-inflammation activities. The subtotal nephrectomy model caused chronic renal failure, inflammation, and severe oxidative stress [38]. In normal conditions, inflammation and stress oxidative upregulated Nrf2, but the lower expression in SN14 and SN28 groups than SO indicated a reduction of activating transcription factor in the remaining kidney [38]. Nrf2 expression in the C14 and C28 groups was confirmed to be significantly higher than SN14 and SN28 [34].



(**a**) (**b**)

Figure 2. (a) qRT-PCR was performed for NFκB. NFκB expression was lower in C14 and C28 groups. (b) RT-PCR was performed for Nrf2. Nrf2 expression was high in the C14 and C28 groups. Chlorogenic acid decreased NFκB and increased Nrf2. *$p < 0.05$ vs SO, #$p < 0.05$ vs SN14, ±$p < 0.05$ vs SN28, ~$p < 0.05$ vs C14.

The higher expression might be associated with CGA's antioxidant and anti-inflammatory effects. The expression of Nrf2 in C28 tends to be lower than in C14. Previous

research revealed that CGA could induce Nrf2 [39,40]. Induction and activation of Nrf2 signaling by pharmacological intervention can be favorable in protecting against inflammation and oxidative stress, which involve kidney dysfunction in AKI and CKD [41].

4. Conclusions

Early chlorogenic acid administration suppresses inflammation by downregulating the expression of pro-inflammatory factors NFκB and CD68 expression and upregulating Nrf2 expression, which involves antioxidant and anti-inflammatory effects. It revealed that chlorogenic acid has anti-inflammatory activity in CKD progression.

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Conflicts of Interest

The authors declare no conflict of interest.

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