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Effects of Yacon Leaf Extract on MCP-1 and IL-10 Expressions and Macrophage Phenotypes in CKD Mouse Model

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Abstract

Macrophages are essential in tissue homeostasis and immunity, but also contribute to disease development and progression. Chronic kidney disease (CKD) is characterized by interstitial infiltration of macrophages, the density of which correlates inversely with kidney survival. Studies have shown that yacon (*Smallanthus sonchifolius*) has beneficial effects on CKD. Therefore, this study aimed to investigate the effects of yacon leaf extract on mice with subtotal nephrectomy by evaluating the M1 and M2 macrophage counts and mRNA expressions of monocyte chemoattractant protein-1 (MCP-1 and IL-10. The mice were randomly divided into five groups: SO (negative control: underwent sham operation), SN (positive control: underwent subtotal nephrectomy), and yacon-treated groups: YK1, YK2, and YK3 (underwent subtotal nephrectomy, given peroral yacon leaf extract for 14 days with doses of 24,5 mg/kgBW/day, 49 mg/kgBW/day, and 98 mg/kgBW/day, respectively). The macrophage subtypes were assessed using immunohistochemistry anti-CD68 for M1 and anti-Arginase I for M2. MCP-1 and IL-10 mRNA expressions were analyzed using semi-quantitative PCR. Results showed that yacon leaf extract could significantly lower the M2 macrophage count ($p<0.001$) and the mRNA expressions of MCP-1 and IL-10 in all yacon-treated groups when compared to the SN group. However, the M1 macrophage count was only lower in the YK2 group ($p=0.009$). In conclusion, the administration of yacon leaf extract could attenuate kidney injury by lowering the macrophage count and the expression of MCP-1 and IL-10.

Keywords: Chronic kidney disease, IL-10, macrophage, MCP-1, yacon

Introduction

Chronic kidney disease (CKD) is a global health problem that has a poor prognosis. The prevalence of kidney disorders is increasing worldwide, leading to a higher number of chronic kidney failures that require costly treatments such as renal replacement therapy and kidney transplantation.[1] The global prevalence of CKD was estimated to be 13.4% in 2019, with more than 4.902 million needing renal replacement therapy.[2]

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Fibrosis is a common final pathway in CKD that causes progressive loss of physiological function. An in-depth understanding of kidney fibrosis can help develop optimum disease management for patients with CKD. CKD can be characterized by interstitial macrophage infiltration, the density of which correlates inversely with kidney survival. Macrophages synthesize and secrete multiple products that can promote fibrogenesis, such as growth factors, cytokines, enzymes, and matrix proteins.[3]

Macrophages are categorized into two subtypes, namely M1 and M2. Pro-inflammatory M1 macrophages function to eliminate pathogens. Macrophage polarization towards M1 can be stimulated by multiple signals such as tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), and monocyte chemoattractant protein-l

(MCP-l).[4] M2 macrophages prevent the spread of tissue damage and induce tissue recovery by the stimulation of interleukin-4 (IL-4), IL-13 immune complex, lipopolysaccharides (LPS), IL-10, and transforming growth factor-β (TGF-β). Therefore, M1/M2 macrophage polarization can be used to determine whether pro-inflammatory or anti-inflammatory processes are present in the kidney microenvironment.[5]

Yacon (*Smallanthus sonchifolius*) is a native plant from the Andes Mountains. It has been shown that yacon administration in patients with CKD can improve plasma glucose and insulin levels, urine albumin and creatinine levels, kidney hypertrophy, tubulointerstitial fibrosis, and the basement membrane. These results indicate that yacon has protective effects against diabetes and kidney injuries related to diabetes.[6]

Yacon leaves are rich in sesquiterpene lactones (STLs), flavonoids, phenolic compounds with chlorogenic acids (CGA), diterpenes, and phenylpropanoids. Chlorogenic acids possess antioxidant and anti-inflammation effects.[7,8] Research by Hwang et al. in 2014 showed that chlorogenic acid could: (1) reduce the production of NO through reducing iNOS levels, (2) suppress the production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, as well as reducing the chemokine CXCL1 (CXC chemokine ligand-1), and (3) inhibiting Ninj1 which is important for leukocyte infiltration.[9]

Phenotype changes of mesangial cells into cells with fibrotic characteristics occur due to oxidative stress. Yacon administration has been proven to prevent mesangial cell matrix expansion and membrane thickening in glomeruli and tubules due to the antioxidant activity of its polyphenol content. This occurs due to a decrease in TGF-β/Smads that contribute to the formation of tissue fibrosis.[6]

To prevent the progression of kidney fibrosis, the development of CKD therapy that targets macrophage infiltration and the chemokines and cytokines involved in macrophage polarization might be beneficial. Therefore, this study aimed to investigate the effects of yacon leaf ethanolic extract on CKD mice model with subtotal nephrectomy by evaluating the number of M1 & M2 macrophages and the mRNA expressions of MCP-1 and IL-10.

Methods

The method of treatment and handling of the experimental animal was conducted following the National Guidelines on Health Research Ethics 2011 established by the Ministry of Health, Republic of Indonesia. The study received ethical approval from the Integrated Research and Testing Laboratory Animal Ethics Committee, Universitas Gadjah Mada (reference number: 00067/04/LPPT/VI/2018). This study was conducted at the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada in 2017.

This study used 25 male mice weighing 30-40 g. The mice were acclimatized at controlled room temperature with the 12-h light-dark cycle for seven days before the experiment with ad libitum access to water and food. The mice were randomly divided into five groups: SO (negative control: undergo sham operation), SN (positive control: undergo subtotal nephrectomy), and the yacon-treated group: YK1, YK2, and YK3 (undergo subtotal nephrectomy, given different doses of yacon leaf extract). Before the procedure, the mice were anesthetized intraperitoneally by pentobarbital 1:10 (0.10/10gBW). Unilateral nephrectomy was performed at the right kidney, and two days after, the superior and inferior poles of the left kidney were sectioned. Laparotomy was performed on mice in the SO group without injuring the kidneys. Rogers et al. (2014) explained that sham surgery, like placebo drugs, serves to neutralize biases such as the placebo effect.[10]

Yacon (*S. sonchifolius*) leaves dried under indirect sunlight were ground and filtered using a 360° mesh filter to attain homogenous powder. The powder was then extracted by 70% ethanol maceration and diluted using aqua dest. This extract was administered to the yacon-treated groups in graded doses once a day, while the SO and SN groups received 1.00% distilled water. The yacon-treated groups, YK1, YK2, and YK3, were given peroral yacon leaf extract for 14 days. The extract is in paste form and dissolved in distilled water before being delivered to mice using an oral probe.

Based on research by Honore et al. in 2012, the dose of yacon that had a renoprotective effect in mice was 70 mg/kgBW.[6] Based on Lauren & Bacharach's 1964 conversion table, 200 grams of rats is identical to 20 grams of mice with a conversion value of 0.14. If 200 grams of rats were given 14 mg of yacon extract, then 20 grams of mice were given 14 mg x 0.14, which was 1.96 mg/20mgBW/day or 98 mg/kgBW/day as the X dose. Based on these results then, the mice were grouped and given Yacon extract with doses of

¼X (24.5 mg/kgBW/day), ½X (49 mg/kgBW/day), and X (98 mg/kgBW/day).

To ensure the rats had entered a CKD state, haemoglobin (Hb), erythrocyte count, and creatinine level measurements were performed on blood drawn from the retro-orbital vein. The blood sample was tested at the Universitas Gadjah Mada Faculty of Medicine's Laboratory of Clinical Pathology. Using a kinetic test without deproteinization, serum creatinine was determined with the Jaffé method (Creatinine FS; DiaSys®, Germany). Proteinuria was quantified using a dipstick (Uriscan® 3 GPH strips; BioSys®, USA). These data were published in Cahyawati et al. (2017) and Purwono et al. (2020).[11,12]

The kidney tissue was extracted using Genezol solution (Geneaid, Taiwan) following the manufacturer's protocol. The total RNA was quantified, and cDNA synthesis was performed using 5 x RT-buffer (Toyobo, Japan), deoxyribonucleotide triphosphate (dNTP) (Takara, Japan), ReverTraAce® (Toyobo, Japan), random primers (Takara, Japan), and Taq Master Mix (Promega, USA). PCR was performed to inspect MCP-1, IL-10, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expressions. The initial denaturation process was performed at a temperature of 94 °C for 2 min. The PCR condition was set for 30 cycles for GAPDH, 35 cycles for IL-10, and 40 cycles for MCP-1, with the following details for each cycle: 94°C for 10 s (denaturation), 60°C for 20 s (annealing), 72°C for 1 min (elongation), and the last extension was 72 °C for 10 min. The primers used were: 1) MCP-1: GGCATCACAGTCCGAGTCACAC (forward) and CTACAGACAACCACCTCAAGCACTTCTGTAG (reverse); 2) IL-10: ATTTGAATTCCCTGGGTGAGAAG (forward) and CACAGGGGAGAAATCGATG-ACA (reverse); 3) GAPDH: TCAACAGCAACTCCCACTCTTCCA (forward) and ACCCTGTTGCTGTAGCCGTATTCA (reverse). The PCR products were then analyzed using gel electrophoresis. The gene expressions were quantified using a densitometry analysis using the ImageJ software version 1.40 and normalized to the GAPDH mRNA expression.

The histological slides that had been deparaffinized were heated in citrate buffer 1x and incubated with 3.00% H202 and blocking solution (Biocare Medical, USA). The slides were then incubated with the anti-CD68 antibody (Abcam, United Kingdom) to identify the M1 macrophages and anti-Arginase 1 antibody (Santa Cruz Biotechnology, USA) for M2 macrophages. The slides were incubated with species-specific secondary antibodies and avidin-biotinylated complex-horseradish peroxidase (Biocare Medical, USA). The histological slides were then observed using a light microscope (Olympus, Japan) with a magnification of 400x. From each histological slide, 15 random fields of view were photographed. ImageJ software performed M1 and M2 cell counts on the histological image. The cell counts were taken two times, and the mean

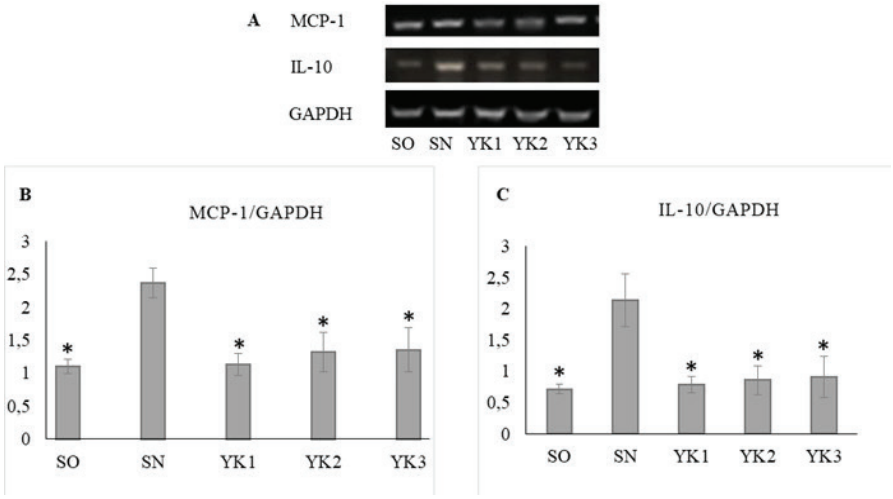

Figure 1 mRNA Expressions of MCP-1 and IL-10 in All Groups (n=5 per group)

A. Gel electrophoresis images of MCP-1, IL-10, and GAPDH mRNA expressions in all groups. B. mRNA expressions of MCP-1. C. mRNA expressions of IL-10. *Statistically significant (p< 0.005) analysis performed using One-Way ANOVA

Table 1 M1 and M2 macrophage cell counts and M1/M2 ratio

Group	M1 (cell)	M2 (cell)	M1/M2 Ratio
SO	46 ± 33*	127 ± 45*	0.4 ± 0.1
SN	195 ± 40	299 ± 47	0.9 ± 0.1
YK1	97 ± 37	116 ± 31*	0.7 ± 0.2
YK2	52 ± 41*	128 ± 48*	0.5 ± 0.1
YK3	99 ± 42	148 ± 25*	0.5 ± 0.1
p-value	$P = 0.004$	$P < 0.001$	$P = 0.490$

Data presented in means±standard deviation; n: number of samples; *significantly different than the SN group ($p<0.05$); analysis was performed using One-Way ANOVA followed by Tukey's post-hoc.

values were used.[13,14]

The data were analyzed using SPSS 23 for Windows. The Shapiro-Wilk test was used to see data normality. All data were normally distributed, so the subsequent analysis was performed using One-Way ANOVA. The p-value <0.05 was used to determine the level of significance. Tukey's post hoc test was used to see differences between groups.

Results

The mRNA expressions of MCP-1 and IL-10 can be seen in Figure 1. The one-way ANOVA test showed differences in MCP-1 mRNA expressions among groups (p=0.043). The SN group showed a significantly higher mRNA expression of MCP-1 than the SO and all yacon-treated groups (YK1, YK2, and YK3) with P values of 0.008, 0.009, 0.029, and 0.012, respectively. The SO group did not show different MCP-1 mRNA expression from the yacon-treated groups. There were also no differences in MCP-1 mRNA expressions among the yacon-treated groups. The mRNA expressions of IL-10 also showed a similar pattern as the MCP-1 ($p<0.001$). The SN group

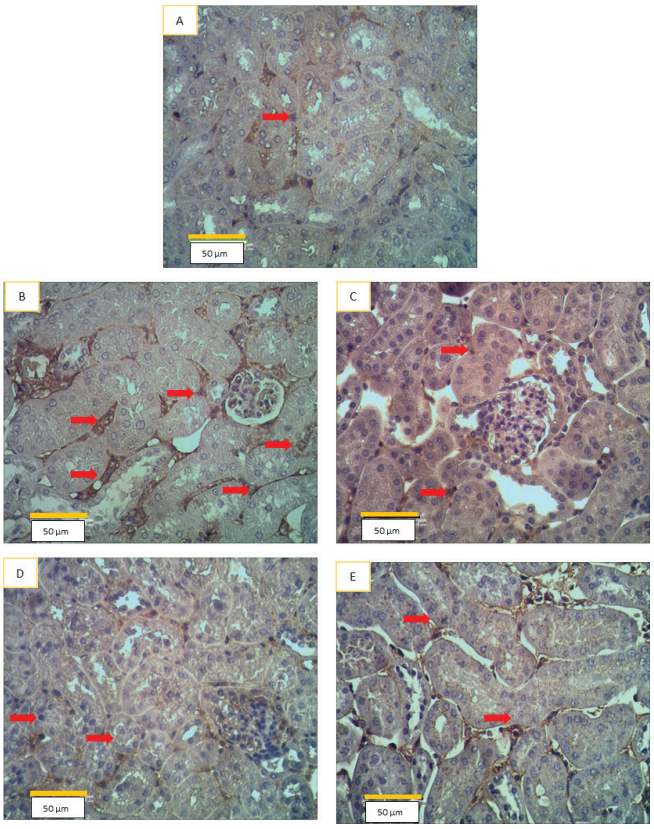

Figure 2. Anti-CD68 Immunohistochemical Staining for Macrophage Detection. Magnification 400 times and scale 50 µm. Information: (A) SO, (B) SN, (C) SN-YK1, (D) SN-YK2, (E) SN-YK3. Red arrows indicate macrophage cells

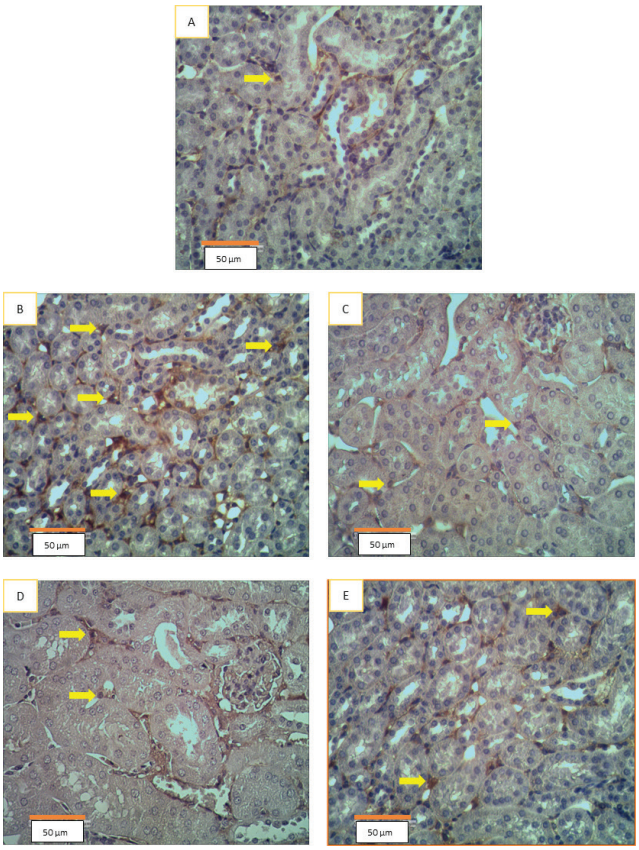

Figure 3. Anti-Arginase 1 Immunohistochemical Staining for Detection of M2 Macrophages. Magnification 400 Times and Scale 50 µm. Information: (A) SO, (B) SN, (C) SN-YK1, (D) SN-YK2, (E) SN-YK3. Yellow Arrows Indicate M2 Macrophage Cells

expressed significantly higher IL-10 than the SO and the yacon-treated groups with P values of <0.001, <0.001, 0.001, and <0.001, respectively. No significant differences were detected between the SO and all yacon-treated groups.

Table 1 presents the number of M1 and M2 macrophages and the M1/M2 ratio between groups while Figure 2 and 3 show the representative immunohistological imaging of the kidney tissue against anti-CD68 (M1) and anti-Arginase 1 (M2) respectively. The number of M1 macrophages in the SN groups was significantly higher than in the SO and YK2 groups, with p-values of 0.006 and 0.009, respectively. However, there were no differences between the SN, YK1, and YK2 groups. A higher number of M2 macrophages was detected in the SN groups than in SO and all yacon-treated groups, with p-values of <0.001, <0.001, <0.001, and <0.001, respectively. There was no significant difference between the SO and the three yacon-treated groups.

All groups' M1/M2 ratios showed no differences (p=0.490). The M1/M2 ratio in the SN group was close to one, indicating that the number of M1 and M2 were equal. Lower M1/M2 ratios were seen in the YK1, YK2, and YK3 groups, showing fewer M1 macrophages than the M2 subtype in the groups administered with yacon leaf extract.

Discussion

Kidney fibrosis is the final common pathway of CKD, which is characterized by extracellular matrix deposition. Subtotal nephrectomy will induce the extracellular matrix deposition, glomerular basement membrane adhesion to the Bowman's capsule, glomerular capillary lumen obliteration, accumulation of proteinous material on capillaries (hyalinosis), and intracellular foam cell (lipid-laden macrophage), all of the hallmarks of glomerulosclerosis.[15]

On the other hand, in mice treated with Yacon herbal ethanolic extract after a 5/6-subtotal nephrectomy has a lower glomerulosclerosis and proteinuria score.[12]

This study investigated the effects of yacon leaf extract on the CKD mice model by examining the mRNA expressions of MCP-1 and IL-10 and the number of M1 and M2 macrophages. The CKD mice model (SN group) showed increased MCP-1 and IL-10 mRNA expression levels and higher numbers of M1 and M2 macrophages. The administration of yacon leaf extract in three graded doses lowered the number of M2 macrophages and the mRNA expressions of MCP-1 and IL-10 in all yacon-treated groups compared to the SN group. However, the number of M1 macrophages was only lower in the yacon-treated group given the 49 mg/kgBW/day dose.

Chronic kidney disease modelling with a subtotal nephrectomy procedure can lower the number of functional nephrons, resulting in glomerulus hyperfiltration and kidney hypertrophy.[16] This condition will also trigger systemic hypertension as well as functional and morphological damage to the remaining kidney, which are the main characteristics of CKD.[17] Fewer nephrons lead to a lower glomerular filtration rate (GFR) and efferent arteriole constriction, increasing hydrostatic pressure of the glomerulus and causing damage to endothelial and mesangial cells. This damage causes aggregation and activation of platelets and cytokine secretions. These sequential events finally lead to the progressive reduction of the remaining nephron and exacerbate kidney failure.[18]

Inflammation and inflammatory mediators can decrease kidney blood flow and GFR through multiple cytokines and the infiltration of inflammatory cells such as T-cells and monocytes. The activation of C-C chemokine receptor type 2 and increased MCP-1 in plasma and kidneys can cause macrophage accumulation and activation.[19] In line with that, increased expression of MCP-1 was detected in the CKD model of this study. MCP-1 is the main factor influencing macrophage accumulation in CKD models in animals and humans. This inflammatory mediator is expressed by the glomerular and tubular epithelial cells. Yacon leaf extract administration was shown to lower the MCP-1 expression. This might be due to its inhibitory effects on the mesangial and tubular cells, as supported by a study by Purwono et al. (2020), reporting that yacon leaf extract could lower glomerulosclerosis and tubular injury.[12] Another study by Widowati et al. (2023) also confirmed the protective effects of yacon against kidney failure by preventing the expansion of matrix mesangial cells as well as the thickening of the glomerulus and tubule membranes.[7]

Macrophages and dendritic cells mainly produce anti-inflammatory IL-10.[12] IL-10 stimulates the over-proliferation of mesangial cells, contributing to the progression of renal failure. This was in line with our findings, where the mRNA expression of IL-10 was higher in the CKD model. The administration of yacon leaf extract lowered the IL-10 mRNA expression.

The expression of IL-10 reflects the number of M2 macrophages in the kidney interstitial. In line with that, the CKD model showed a high number of M2. An increased number of M2 macrophages leads to higher production of TGF-β1 that facilitates kidney fibrosis by inducing epithelial-to-mesenchymal transition (EMT). The adoptive transplantation of M2 macrophages was reported to exacerbate kidney fibrosis.[21] The administration of yacon leaf extract managed to lower the number of M2 macrophages. This suggests that yacon leaf extract might have an inhibitory effect on macrophage polarization towards the M2 subtype. As a result, decreased M2 macrophages will inhibit the formation of kidney fibrosis through EMT inhibition. M2 macrophages also release anti-inflammation mediators, such as IL-10 and TGF-β, which can suppress inflammation.[22] Thus, to achieve the condition of homeostasis, the number of M2 macrophages released must be higher than that of the M1.

This study found that the M1/M2 ratio of the CKD model is close to one, indicating that there were almost equal numbers of both M1 and M2 subtypes in the kidney interstitial. This ratio was higher than all other groups, where they had a higher number of M2 than the M1 macrophages. Yacon leaf extract lowered the number of M2 macrophages in all yacon-treated groups. However, it could only reduce the number of M1 in the group given the 49 mg/kgBW/day dose, suggesting that this dose effectively lowered the number of both macrophage subtypes.

The infiltration of M1 and M2 macrophages, as well as local proliferation from both types of cells, correlates with the degree of tubulointerstitial damage severity and the decreasing physiological function of the kidney. The results of the present study confirmed that the administration of yacon leaf extract decreased the expression of MCP-1 and IL-10 involved in the interstitial infiltration of inflammatory cells and reduced

the number of M1 and M2 macrophages in the CKD mice model. It is expected that fibrosis can be avoided by controlling inflammation and tissue recovery processes within the kidney, and the progressivity to CKD can be slowed down.

This study has limitations. The 14 days of yacon leaf administration might not have been long enough to lower the number of M1. A more extended treatment period might be helpful in determining the effectiveness of all given doses. This study also did not examine the contents of the yacon leaf extract, so it could not pinpoint the exact components of the extract responsible for the processes described in this study. The use of semi-quantitative PCR in measuring MCP-1 and IL-10 mRNA expressions may not be as reliable as real-time PCR.

In conclusion, this study showed that the administration of yacon leaf extract could attenuate kidney injury by lowering the number of macrophages and the expression of inflammatory mediators such as MCP-1 and IL-10. Further studies on this topic should be conducted better to understand the roles of yacon in kidney diseases and develop it as a potential therapeutic agent.

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