ORIGINAL ARTICLE

Yacon Extract Attenuated Kidney Fibrosis in 5/6-subtotal Nephrectomy Mouse Model by Upregulating HGF and BMP-7 mRNA Expression

Setyo Purwono[1,4], Abdur Rahman Faqih Al Jundi[2], I Made Kawiyasa[2], Alfina Meidina Rizka Cahyatika[2], Andika Putra Cipta[2], Galih Asa Andrianto[2], Rendy Candra[2], Dwi Cahyani Ratna Sari[3], Fajar Dwi Astarini[5], Eryna Ayu[3], Nugra Desita[3], Nur Arfian[3,4]

[1] Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2] Undergraduate student, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3] Department of Anatomy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[4] Herbal Medicine Unit, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[5] Master Program in Biomedical Sciences, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

ABSTRACT

Introduction: Chronic kidney disease (CKD) leads to tubular injury, kidney fibrosis and anemia. These conditions are influenced by fibrotic and anti-fibrotic substances, such as Transforming Growth Factor beta-1 (TGF-β1), Hepatic Growth Factor (HGF), and Bone Morphogenic Protein-7 (BMP-7). Yacon is an herbal medicine which has not been elucidated in CKD. This study aimed to investigate the effect of ethanolic extract of Yacon leaves on attenuating renal injury in CKD model in mice. **Methods:** We performed 5/6 subtotal nephrectomy (SN) in male Swiss-Webster mice (3 months old, 30–40 grams) to induce chronic kidney disease, then the mice were sacrificed at day 14. The mice (n=25) were divided into five groups: one SN group, three groups of SN with administration of Yacon extract, and one group of sham operation (SO, with supplementation of 0.1% aquadest). There were three different doses of ethanolic extract of Yacon leaves: 98 mg/kg BW (SN+YK1), 49 mg/kg BW (SN+YK2), and 24.5 BW mg/kg (SN+YK3). Tubular injury, perivascular and interstitial fibrosis were quantified based on histopathological examination. Reverse-transcriptase PCR (RT-PCR) was performed to quantify HGF and BMP-7. **Results:** SN group demonstrated CKD with elevation of creatinine level, anemia, tubular injury, glomerulosclerosis, and fibrosis. Yacon extract treatment showed attenuation of injury with lower creatinine level, tubular injury, glomerulosclerosis and fibrosis compared to the SN group. *HGF* and *BMP-7* mRNA expressions were higher in Yacon-treated groups than the SN group. **Conclusion:** Yacon treatment might ameliorate CKD through reducing fibrosis and increasing expression of anti-fibrotic genes.

Keywords: Chronic Kidney Disease, Yacon, Kidney Fibrosis, *HGF*, *BMP-7*

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

INTRODUCTION

The increase of non-communicable diseases has recently reached 60% of the global mortality. It becomes one of the biggest obstacles in achieving the Sustainable Development Goals (1). Chronic kidney disease is one of the global health issue. The increasing incidence and prevalence in the world has been linked to the increasing risk of morbidity and mortality, and directly causing the increase of financial burden. According to the WHO, the disease involving kidney and urinary tract causes the mortality of 850,000 people every year and ranks the twelfth for the mortality. Survey done by the Indonesian Nephrology Association in 2009 revealed that 18 million Indonesian suffered from the chronic kidney disease. According to Kidney Care Foundation in Indonesia, there were 40,000 people suffering from chronic kidney disease (2).

Chronic kidney disease is caused by several factors such as diabetes mellitus, hypertension, infection, and urinary tract obstruction. The condition will increase the intraglomerular pressure, the filtration barrier permeability, endothelial cell dysfunction, activation of mesangial cells, podocytes, and tubular cells, synthesis of extracelullar matrix, proteinuria/albuminuria, and the decrease of glomerular filtration rate (GFR). The increase of extracellular matrix is the cause of kidney interstitial fibrosis (3).

Kidney interstitial fibrosis is a common irreversible pathological hallmark in chronic kidney disease, including nephropathy which causes End Stage Renal Disease (ESRD). Kidney fibrosis process is classified into two forms, glomerulosclerosis and kidney tubulointerstitial fibrosis. Kidney tubulointerstitial fibrosis develops into ESRD. Cellular events in tubulointerstitial fibrosis include the infiltration of a huge number of inflammatory cells, activation and expansion of fibroblast, production and deposition of extracellular matrix components, tubular atrophy and microvascular depletion. These events cause the destruction of kidney parenchyma and loss of kidney function (4).

As the fibrosis happens, anemia also occurs on the patients with chronic kidney disease by causing the depletion of erythropoietin (EPO). EPO is the hormone produced by the resident fibroblasts. In response to injury, resident fibroblasts transdifferentiate into myofibroblasts that reduce the production of EPO (5). Nistala et al., 2009 showed that perivascular fibrosis decreases along with the reduction of oxidative stress. According to the existing therapy for kidney fibrosis, there is a need to provide an alternative therapy to inhibit the progression of kidney fibrosis through the intervention of the effect of oxidative stress (6).

Yacon (*Smallanthus sonchifolius*) herbal leaves have antioxidant effect in phenolic acid compound such as chlorogenic acid, cafeic acid, choumaric acid, photocatecuic acid and flavonoid compound (7). The flavonoid compound contained in Yacon leaves is known for its inhibiting activity against Angiotensin Converting Enzyme (ACE), an enzyme that has important role in regulating Renin-Angiotensin System (RAS) (8).

The Yacon herbal leaves are potential to inhibit the progression of glomerulosclerosis by inhibiting the production of RAS and reactive oxygen species (9). However, there has not been yet any research about the effect of Yacon leaves on glomerulosclerosis and proteinuria.

MATERIALS AND METHODS

Animal experiment

We used 30 adult (three months old) 30-40 grams male Swiss-Webster mice, obtained from Animal Model Care Unit, Universitas Gadjah Mada (Yogyakarta, Indonesia) in this study. The mice were randomized and maintained under standard laboratory conditions (at 22+20C; 50±5% humidity; 12/12 h light/dark cycle) with access ad libitum to the animal diet and tap water. All experimental procedures were conducted according to the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Universitas Gadjah Mada-Dr. Sardjito General Hospital.

5/6 Subtotal Nephrectomy procedure and administration of the extract of Yacon leaves

The 5/6 subtotal nephrectomy (SN) procedure was performed to induce chronic kidney disease (CKD) in mice. After injecting 0.1 mL/10gBW pentobarbital intraperitoneally, we performed uni-nephrectomy of the right kidney, which was then continued with removing superior and inferior polus of the left kidney one day after the uni-nephrectomy. Sham operation procedure was carried out in the SO group by opening the abdomen without performing the 5/6 SN procedure.

Yacon extract was administered orally to the three SN groups starting from one day after the SN procedure. The extract was given for 14 days until the mice were sacrificed. The administration dose of Yacon extract was based on the conversion of the dose given to rat in diabetes mellitus model (10). We divided the Yacon extract into 3 doses (one dose for each group): 98 mg/kgBW (YK-1 group, n=6), 49 mg/kgBW (YK-2 group, n=6), and 24.5 mg/kgBW (YK-3 group, n=6).

Yacon leaf solution preparation

We used Yacon leaves which were obtained from the mountain area of Wonosobo, Central Java, Indonesia. The leaves were then identified and authenticated at the Faculty of Pharmacy (Laboratory of Pharmacognition), Universitas Gadjah Mada. The Yacon leaves were then macerated with 70% ethanol at the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada.

Biochemistry analysis

Blood from the retro-orbital vein was taken for measurement of creatinine level, erythrocyte count and hemoglobin (Hb) level. The blood sample was examined at the Laboratory of Clinical Pathology, Faculty of Medicine Universitas Gadjah Mada. Serum creatinine was measured with kinetic test without deproteinization according to the Jaffй method (Creatinine FS; DiaSys®, Germany). Proteinuria was measured with dipstick (Uriscan® 3 GPH strips; BioSys®, USA).

Histopathological examination for tubular injury and fibrosis

At the end of the study, the mice were given a general anesthesia with a 0.1 mL/gBW intraperitoneal injection of sodium pentobarbital. Then, the mice were dissected so that we could harvest the left kidneys. The kidney was cut into two parts: the anterior part for histopathological examination, and the posterior part for RNA extraction.

The anterior part of renal tissue was embedded in paraffin for assessment of glomerulosclerosis, tubular injury, and immunohistochemical studies. Paraffin-embedded renal tissue was deparafinized using standard sequential techniques, and 4 μm-thick sections were stained with

Periodic Acid-Schiff (PAS). Morphological measurement was conducted blindly by two observers.

The extent of glomerulosclerosis (GS) was graded from 0 to 4 with a semiquantitative score based on the extent of glomerular damage (sclerosis), capillary loops and synechia between glomerular capillaries and the Bowman's capsule (0, normal; 1, mesangial expansion/ sclerosis involving <25% of the tuft; 2, moderate GS (25 to 50%); 3, severe GS (50 to 75%); and 4, diffuse GS involving >75% of the glomerular tuft). For each kidney, the sum of the results for 20 glomeruli was defined as the glomerulosclerosis index (GSI). The GSI of each mouse was calculated as a mean value of all the glomerular scores obtained.

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 3 (0, no change; 1, changes affecting <25% of the section; 2, changes affecting 25 to 50% of the section; and 3, changes affecting 50 to 100% 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.

Perivascular fibrosis score and interstitial fibrosis area fraction quantification

Perivascular fibrosis score was quantified as a ratio of perivascular fibrosis area to vessel area. Quantification was performed based on Sirius Red staining. Red color which surrounding intra renal arteries represented perivascular fibrosis (adventitial layer). Image J software was used for assessment of ten intrarenal arteries. Interstitial fibrosis area fraction assessment was performed based on area fraction of red color positive staining of Sirius red staining from ten fields with 400x magnification. Quantifications were done using Image J software (NIH).

RNA Extraction and cDNA Synthesis

We used 1000 ng RNA from tissue obtained from the posterior part of the left kidney of the mice, which was extracted using on RNAisoplus (Takara, Cat. No. 9109) followed by synthesis of cDNA, using Rever Tra Ace® (Toyobo, Japan, Cat. No. TRT-101) and random primer (Takara, Japan, Cat. No. 3801), with the following PCR conditions: 30°C for 10 minutes (denaturation), 42°C for 60 minutes (annealing) and 99°C for 5 minutes (extension).

Reverse Transcriptase-Polimerase Chain Reaction (RT-PCR) of *HGF* and *BMP-7*

Reverse Transcriptase-PCR (RT-PCR) was carried out to amplify the following specific cDNAs: *BMP-7* (F: 5′-CGAGACCTTCCAGATCACAGT-3′ and R: 5′-CAGCAAGAAGAGGTCCGACT-3′), *HGF* (F: 5′-CATTCAAGGCCAAGGAGAAG-3′ and R: 5′-AACTGGATGTTTGGGTCAG-3′), and GAPDH (F: 5′-TCACCATCTTCCAGGAGCG-3′ and (R: 5′-CTGCTTCACCACCTTCTTGA-3′).

RT-PCR was performed by mixing 2 µL of RT reaction product (cDNA), 12.5 µL of Tag master mix (Bioron, Germany, Cat. No. S101705), 0.6 µL of forward primer, 0.6 µL of reverse primer and 9.3 µL of PCR water. The cDNAs were amplified according to the following conditions: 94°C for 2 seconds (initial denaturation), 94°C for10 seconds (denaturation), 60°C for 20 seconds (the annealing temperature varied for each pair of primers), 72°C for 1 minutes (extension), and 72°C for 10 minutes (last extension). The number of cycles was redetermined for each pair of primers in order to avoid the PCR plateau phase. The PCR products were analyzed in 2% agarose gel along with a 100 bp DNA ladder (Bioron, Germany, Cat. No. 306009).

The gene expressions were quantified through a densitometry analysis using the ImageJ software version 1.40. GAPDH expression was used to normalize the expression.

Statistical Analysis

The results are expressed as mean ± SEM. Multiple comparisons among the groups were done by one-way analysis of variance (ANOVA) and followed by the post hoc Tukey's test.The level of statistical significance was $p<0.05$.

RESULTS

Yacon treatment attenuated glomerulosclerosis, tubular injury and anemia

Histopathological analysis showed renal injury with glomerulosclerosis and tubular injury in SN group. SN group represented not only glomerulosclerosis with the increase of matrix deposition, capillary loop reduction, synechia and mesangial expansion (Fig 1), but also tubular injury with intraluminal cast formation, brush border loss, epithelial cells effacement and inflammatory cells expansion (Fig 1B). Assessments of tubular injury and glomerulosclerosis score were significantly higher in the SN group compared to the SO group (Fig. 1C-D). Yacon-treated groups demonstrated amelioration of glomerulosclerosis and tubular injury with significantly lower glomerulosclerosis and tubular injury score compared to the SN group (Fig. 1C-D). Measurement of Hb level also showed significant lower of Hb level and erytrocyte count in the SN group compared to the SO group (Fig 1E-F), meanwhile Yacon-treated groups had significantly higher Hb level and erythrocyte count compared to the SN. YK-1 group with highest dose of Yacon extract represented the best condition with the lowest glomerulosclerosis and tubular injury score among the Yacon-treated groups. Based on PAS staining, YK1 group had amelioration of tubular injury with brush

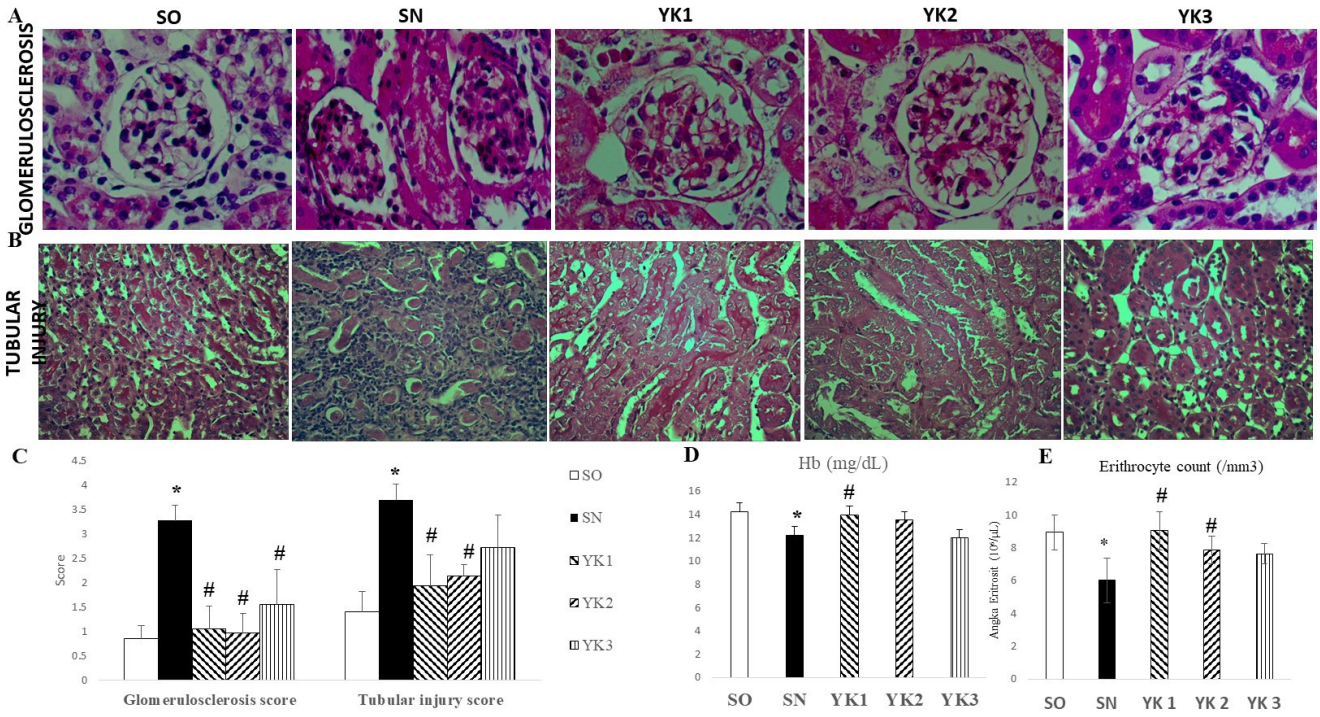

Figure 1:Yacon treatment attenuated glomerulosclerosis and tubular injury in CKD model.(A). Representative pictures of glomerulosclerosis showed matrix expansion, capillary loss and synechia in SN group. (B). Representative staining of tubular injury based on PAS staining showed characteristic of tubular injury such as intraluminal cast formation, inflammation, brush border loss and tubular atrophy in SN group. (C). Assessment of glomerulosclerosis and tubular injury score based on histhopathological appearance in PAS staining. (D). Hemoglobin level quantification. (E). Erythrocyte count quantification. *p<0.05 VS SO group. #p<0.05 VS SN group.

border formation and epithelial cells regeneration. We also found that there was no significant difference in Hb level and erythrocyte count between SO and YK-1 groups.

Yacon treatment attenuated perivascular and interstitial fibrosis

Moreover, we assessed both perivascular and interstitial fibrosis. Sirius Red staining revealed significantly higher perivascular fibrosis score in the SN group compared to the Yacon-treated groups. Meanwhile, only YK1 group showed significantly lower perivascular fibrosis score compared to the SN group (Fig. 2A & C). SN group also had significantly higher interstitial fibrosis area fraction compared to the SO group. Attenuation of fibrosis was shown by significantly lower area fraction of interstitial fibrosis in Yacon-treated groups. YK1 group demonstrated the lowest interstitial area fraction among the Yacon-treated groups. We did not find significant difference between SO and YK1 groups (Fig. 2B & D).

Yacon treatment induced upregulation of antifibrotic genes

RT-PCR analysis revealed downregulation of antifibrotic genes (*BMP-7* and *HGF*) in SN group as shown in Figure 3. The SN group had significantly lower mRNA expression of *HGF* compared to the SO group. However, we did not find any significant difference of mRNA expression of *BMP-7* between SN and SO groups, although expression of *BMP-7* tended to be lower in SN group. The mRNA expression of *HGF* in Yacon-treated groups was significantly higher compared to the SN group. It was also associated with significantly higher *BMP-7* mRNA expression in Yacon-treated groups compared to the SN group. YK-1 group had the highest *HGF* mRNA expression.

DISCUSSION

This study demonstrated the renoprotective effects of Yacon extract in CKD model in mice which might be associated with upregulation of anti-fibrotic expression, *HGF* and *BMP-7*. SN induced glomerulosclerosis, which was characterized by the adhesion of glomerular basement membrane to the Bowman's capsule, extracellular matrix deposition, the obliteration of glomerular capillary lumen and the accumulation of proteinous material on capillary (hyalinosis) and intracapillary foam cell (lipid-laden macrophage) (11). On the other hand, treatment of Yacon herbal ethanolic extract on the mice model with 5/6-subtotal nephrectomy could inhibit the development of glomerulosclerosis. This was shown by the lower glomerulosclerosis score in the Yacon-treated groups compared to SN group (Fig. 1).

Mechanical stress was known to have an important role towards the progression of glomerulosclerosis. The hyperfiltration was caused by the defect in the filtration barrier. It resulted in the increase of single-nephron

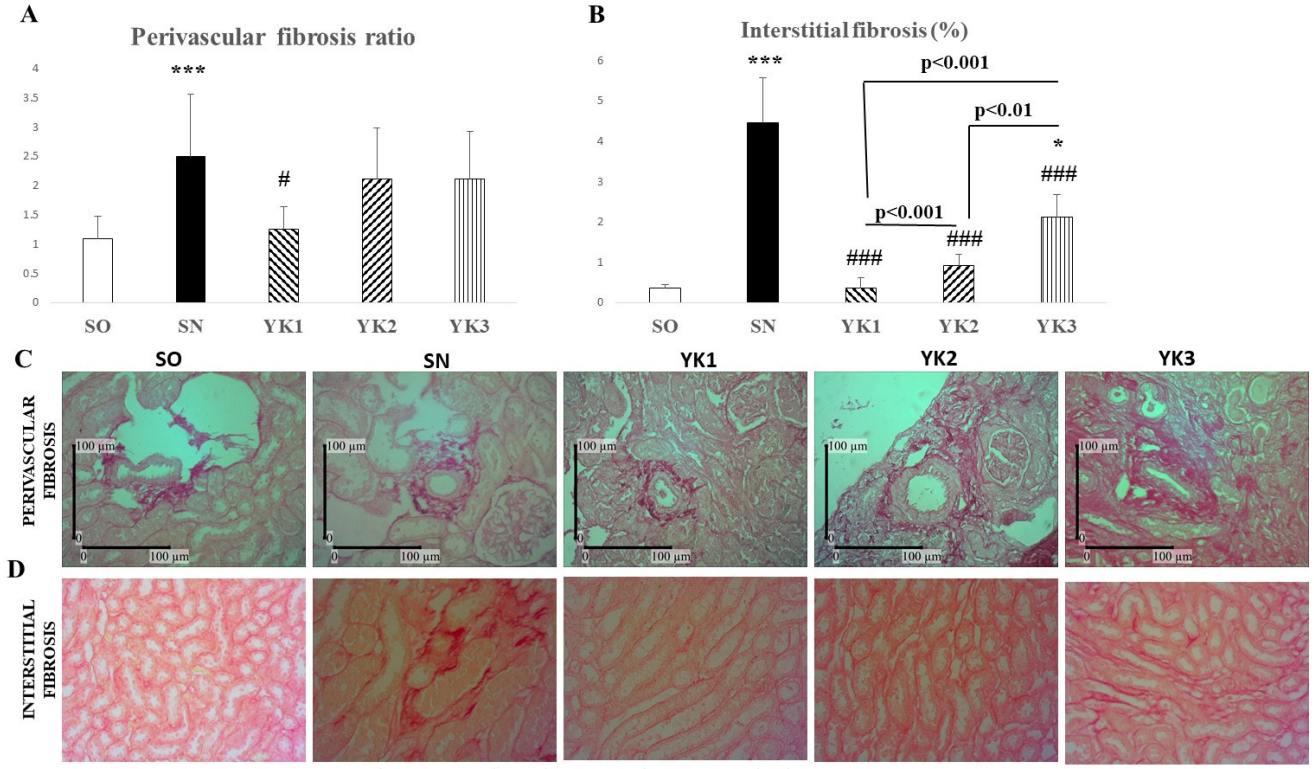

Figure 2: Yacon treatment attenuated perivascular fibrosis and interstitial fibrosis. (A-B). Quantification of perivascular fibrosis score and interstitial fibrosis area fraction. (C). Representative picture of perivascular fibrosis in intra-renal arteries. (D). Representative picture of fibrosis based on Sirius red staining. Fibrosis was collagen deposition with red color. Bar=100 µm. *p<0.05 VS SO group; ***p<0.001 VS SO group. #p<0.05 VS SN group; ###p<0.001 VS SN group.

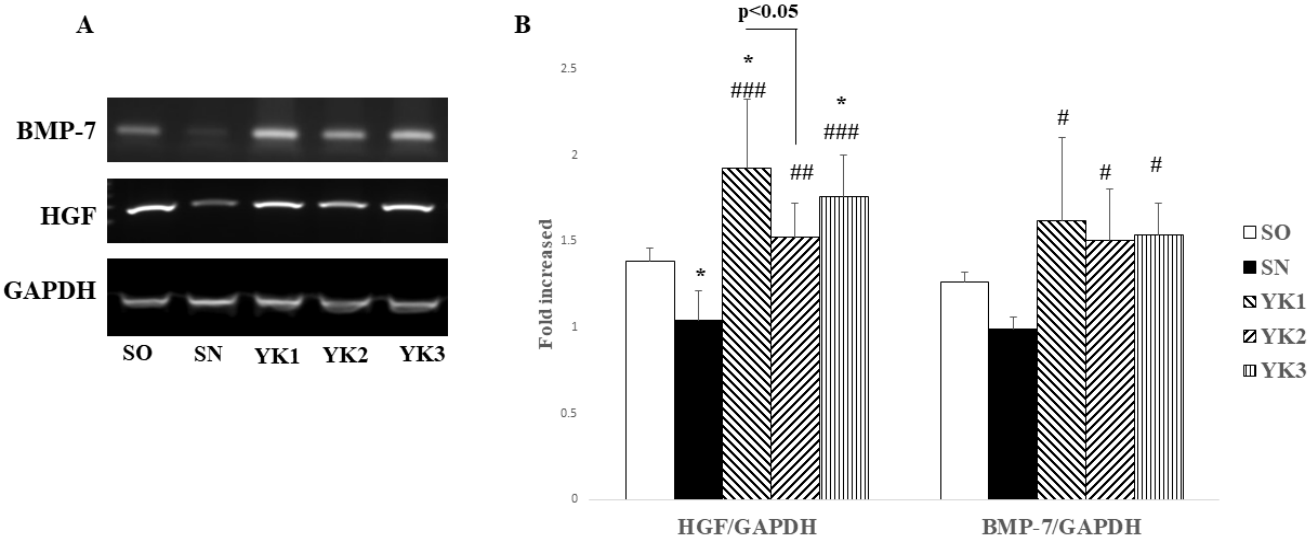

Figure 3: Yacon treatment upregulated *HGF* and *BMP-7* mRNA expression as anti-fibrotic genes. (A). Representative gel electrophoresis results from RT-PCR of *HGF* and *BMP-7* mRNA. (B). Densitometry analysis of RT-PCR results.

glomerular filtration rate (SNGFR) and hypertrophy of glomerulus. Hypertrophy of glomerulus caused imbalance between GBM and worsened the decreasing number of the podocytes, resulting in further injury (12). Through this mechanism, the production of Reactive Oxygen Species (ROS) was induced through Angiotensin II pathway and contributed to the renal injury including the progression of glomerulosclerosis. On the other hand, the upregulation of TGF-β resulted in structural injury and disturbing the integrity of podocytes. TGF-β also induced the proliferation of mesangial cells and the increase synthesis of the extracellular matrix. The progression of glomerulosclerosis was shown in the presence of synechia between glomerular capillary and Bowman's capsule. The presence of synechia provided an opening for the parietal epithelial cells migrating to

glomerular capillary. Therefore, the migrating parietal epithelial cells would deposit in the matrix and caused the appearance of sclerotic lesion (13).

Glomerulosclerosis caused tubular injury in chronic kidney disease (14). Tubular injury was the terminal condition of epithelial cells of the kidney tubulus, shown by intratubular cast found in the lumen of kidney tubules (15). Glomerular hypertrophy and intraglomerular hypertension also contributed to the renal tubulointerstitial injury. The high pressure and flow in afferent artery caused the endothelial dysfunction. Thrombosis occured due to the activation and adhesion of the leukocytes on subintima. Hypertension also caused the production of fibrinoid necrosis on the vessel wall. It then caused ischemia and hypoperfusion. The distribution pattern of peritubular microcirculation was very unique as the circulation in efferent artery of glomerulus did not only cause the atrophy of nephron but also the kidney tubulus (16,17).

Kidney fibrosis is the final common pathway of CKD which is characterized by extracellular matrix deposition. The deposition of extracellular matrix in the interstitial fibrosis was known due to the role of myofibroblasts. The production of myofibroblasts was the key factor of chronic kidney disease (18). Here, we demonstrated perivascular and interstitial fibrosis in SN group (Fig. 2). Several factors were thought to contribute to interstitial fibrosis, such as oxidative stress and inflammation (19). However, the interstitial fibrosis process was dominated by TGF-β, Angiotensin II, Nuclear Factor κB (NFκB), and TNF-α produced by kidney tubular and interstitial cells, and macrophage (20). Yacon-treated groups revealed lower perivascular fibrosis score and interstitial fibrosis area fraction compared to SN. This finding may show anti-fibrotic effect of Yacon extract. This was in line with the result shown by Honore et al (2012) that Yacon extract was protective to the kidney injury in diabetic nephropathy induced by the TGF-β/Smads signal-mediated activity (8).

The presence of fibrosis was proven by increase level of fibrosis marker, such as TGF-β1. TGF-β1 cytokine was responsible to the underlying of the progressiveness of kidney disease, such as myofibroblast formation (21). Proliferation and expansion of renal fibroblast may occur due to ROS production during kidney injury, and induces tubular epithelial cell apoptosis (20). Yacon extract has been known as source of anti-oxidant capacity from herbal medicine in diabetic condition (9,22). Thus, Yacon seem to has antifibrotic due to its antioxidant effect also. Fibroblast, as EPO producting cells, undergoes transtition to myofibroblast thus reducing EPO production (23). SN model in this study also induced anemia with significant lower hemoglobin level and erythrocyte count (Fig. 1).

We concluded that attenuation of anemia may associate with amelioration of fibrosis in the Yacon-treated groups. Here we also showed that renoprotective effects of Yacon may relate to upregulation of anti-fibrotic substances, such as *HGF* and *BMP-7* (Fig. 3). During fibrogenesis, anti-fibrotic factors are produced as a respond to TGF-β1. A number of studies documented *HGF* and *BMP-7* as kidney anti-fibrotic factors (24,25). *HGF* counteracts the action of TGF-β1 by inhibition of TGF-β1-mediated myofibroblast activation and extracellular matrix deposition (26). Furthermore, *BMP-7* is a member of TGF-β superfamily which plays anti-fibrotic action by blocking the TGF-β1/Smad3 pathway (25,27). *HGF* and *BMP-7* are well studied as kidney anti-fibrotic factors (2,3). Inflammatory cytokines, growth factors, and prostaglandin that are produced during kidney damage influence the expression of *HGF* (28).

CONCLUSION

In conclusion, this study revealed renoprotective effects of Yacon extract supplementation, which might be modulated by upregulation of anti-fibrotic substances, such as *HGF* and *BMP-7*.

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