Chlorogenic acid attenuates kidney fibrosis via antifibrotic action of BMP-7 and HGF

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ABSTRACT

Background: Kidney fibrosis, characterised by tubulointerstitial fibrosis, is a histological landmark of chronic kidney disease. The body attempts to compensate for progressive detrimental process of kidney fibrosis by producing antifibrotic substances, such as bone morphogenetic protein-7 (BMP-7) and hepatocyte growth factor (HGF). Chlorogenic acid is known to have renoprotective and antifibrotic properties. This study aims to evaluate the effect of chlorogenic acid on unilateral ureteral obstruction (UUO)-induced kidney fibrosis mice model.

Methods: This study was a quasi-experimental with posttest-only control group design. Twenty-five adult male Swiss Webster mice were randomly divided into five groups: sham-operated group (SO), UUO-control day-7 (U7), UUO-control day-14 (U14), UUO-chlorogenic acid day-7 (UC7), and UUO-chlorogenic acid day 14 (UC14). Myofibroblasts were identified by immunohistochemical staining of alpha-smooth muscle actin (α-SMA) while collagen fibers were identified by Sirius Red staining. Both data were presented as area fraction. BMP-7 and HGF mRNA expressions were assessed by reverse transcription PCR (RT-PCR). Data were quantified using ImageJ software.

Results: UUO-control groups (U7 and U14) showed higher α-SMA-immunopositive (6.52±1.33, 18.24±1.39 vs. 0.22±0.01; p<0.05) and Sirius Red-positive area fractions (6.61±0.8, 12.98±2.31 vs. 0.62±0.10; p<0.05), lower BMP-7 (1.02±0.47, 1.18±0.65 vs. 2.09±0.87; p<0.05) and HGF mRNA expressions (1.06±0.31, 0.89±0.14 vs. 1.88±0.81; p<0.05) compared to SO group. UUO-chlorogenic acid groups (UC7 and UC14) showed lower α-SMA-immunopositive (1.24±0.37, 4.58±0.61; p<0.05) and Sirius Red-positive area fractions (4.76±1.03, 3.72±0.54; p<0.05), higher BMP-7 (1.84±0.49, 2.19±0.43; p<0.05) and HGF (1.58±0.38; p>0.05, 1.84±0.42; p<0.05) mRNA expressions compared to UUO-control groups. UUO-chlorogenic acid groups showed BMP-7 and HGF mRNA expressions that were not significantly different from the SO group.

Conclusion: Chlorogenic acid administration prevents kidney fibrosis in UUO mice model through modulating antifibrotic pathway.

KEY WORDS:
Kidney fibrosis, unilateral ureteral obstruction, chlorogenic acid, antifibrotic, BMP-7, HGF

INTRODUCTION

Chronic kidney disease (CKD) is one of the global public health issues in the 21st century. Currently, more than 20 million people in the United States suffer from this disease.[1] Kidney failure, as the end-stage of CKD, is also becoming a serious problem in developing countries over the last 20 years.[2] In Indonesia, CKD was the 27th leading cause of death in 1990 and increased to the 18th position in 2010.[3]

CKD is caused either by structural damage or decreased renal function.[4] Structural damage in CKD is particularly kidney fibrosis. Kidney fibrosis is characterised by the formation of connective tissues caused by accumulation of extracellular matrix components, including type I, III, and IV collagens, proteoglycans, and fibronectins. Interstitial fibroblasts are also transformed into myofibroblasts in kidney fibrosis.[5] Unilateral ureteral obstruction (UUO) mice model is well known to induce kidney fibrosis which begins in the period between days-7 and 14, as indicated by increased expression of α-smooth muscle actin (α-SMA) on days 2, day 4 to day 10.[6]

Kidney fibrosis is primarily mediated by transforming growth factor-β1 (TGF-β1).[7] TGF-β1 activity has the opposite effect to that of bone morphogenetic protein-7 (BMP-7). There is an increased activity of TGF-β1 and decreased activity of BMP-7 in kidney fibrosis.8 While there is a decrease in the BMP-7 expression in injured kidney, there is an increase in the BMP-7 expression during regeneration process.[8] Endogen antifibrotic factors are also released during kidney injury, one of which is hepatocyte growth factor (HGF).[9] HGF has mitogenic, antifibrotic, and antiapoptotic activities on kidney epithelial cells which promotes regeneration and prevents initiation and progression of kidney fibrosis.[10] HGF is also reported to suppress TGF-β1 expression in kidney fibrosis.[11]

Chlorogenic acid has protective and antioxidative effects. Several studies have demonstrated an association between fibrotic mediators and chlorogenic acid.[12] Chlorogenic acid can also suppresses inflammation by inhibiting toll like

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receptor 4 (TLR4).[13] To the best of our knowledge, there is no study on the antifibrotic effect of chlorogenic acid in UUO-induced kidney fibrosis mice model. This study aims to evaluate the effect of chlorogenic acid administration on α-SMA-immunopositive and Sirius Red-positive area fractions, BMP-7 and HGF mRNA expressions in kidney fibrosis.

MATERIALS AND METHODS

Animals

This study was a quasi-experimental with posttest-only control group design. In all 25 adults male Swiss Webster mice with 30-40gram body weight were used, five mice per group. Mice underwent acclimation to laboratory conditions for 3-5 days. Mice were group housed in a 12-hour light-dark cycle at room temperature and given access to food and water *ad libitum*. All procedures using live animals was granted permission from the Medical Research Ethics Committee, Faculty of Medicine, Universitas Tadulako No. A.0940/UN28.1.30/KL/2018.

Unilateral Ureteral Obstruction (UUO)

UUO procedure was performed by ligating the right ureter to induce kidney fibrosis. Mice were anesthetised with ketamine-xylazine-acepromazine cocktail (0.1mL/10g body weight). The right flank region was surgically opened, then the right ureter was visualised. Double ligation at the ureteropelvic junction of the right ureter was performed and then cut between the ligations. Sham-operated group underwent the same procedure except for the ureteral ligation.

Chlorogenic Acid Administration

Mice were divided into five groups which were SO control group, UUO-control day-7 (U7), UUO-control day-14 (U14), UUO-chlorogenic acid day-7 (UC7), and UUO-chlorogenic acid day-14 (UC14). Chlorogenic acid (Sigma Aldrich C3873-1G) was dissolved in aquadest and given to UC7 and UC14 groups at a dose of 14 mg/kgBW/day by intraperitoneal injection for 7-14 days.[14] SO and UUO-control groups were only delivered aquadest via intraperitoneal injection.

α-SMA Immunohistochemical Staining

For sacrifice, the mice were deeply anesthetised and transcardially perfused with 0.9% NaCl. Right kidney was harvested and divided into two parts. Half of the kidney was immersed in RNAlater for RNA extraction and the other half was fixed in 4% PFA in PBS for 24 hours at 4°C, then embedded into paraffin blocks. Four µm-thick sections were prepared for immunohistochemical staining. Slides were taken from 4°C refrigerator and placed in room temperature for 30 minutes, then deparaffinised in xylene and rehydrated in a graded ethanol series. Antigen retrieval procedure was performed by heating the slides in citrate buffer for 15 minutes, then placed in room temperature for 30 minutes. After being washed three times in PBS, the slides were immersed in blocking buffer using Background Sniper (Starr Trek Universal HRP Detection Kit, Biocare Medical) for 20 minutes. The slides were then incubated with anti-α-SMA antibody (Sigma-Aldrich; 1:500 dilution) for overnight at 4°C.

On the second day, the slides were taken from the 4°C refrigerator and placed in room temperature for 30 minutes. After being washed three times in PBS, the slides were immersed in Trekkie Universal Link (Starr Trek Universal HRP Detection Kit, Biocare Medical) for 60-90 minutes at room temperature. The slides were then immersed in TrekAvidin-HRP Label (Starr Trek Universal HRP Detection Kit, Biocare Medical) for 45 minutes at room temperature after being washed three times in PBS. The slides were reacted in 1:400 dilution of DAB solution for 3 minutes in the dark room. The slides were washed in tap water for 5 minutes, counterstained with hematoxylin for 2 minutes and then were dehydrated in a graded ethanol series, cleared in xylene, and coverslipped with Entellan solution (Merck Millipore).

Sirius Red Staining

Paraffin-embedded tissues were sectioned with a thickness of 4µm and then deparaffinised in xylene and rehydrated in a graded ethanol series. After being washed three times in PBS, the slides were immersed in Sirius Red working solution consisting of 1mL Sirius Red and 9mL picric acid for one hour. The slides were then dehydrated in a graded ethanol series, cleared in xylene, coverslipped with Entellan solution, and incubated for 24 hours.

Myofibroblast and Fibrosis Area Fractions

Myofibroblast area fraction was defined as an α-SMA-immunopositive area while fibrosis area fraction was defined as a Sirius Red-positive area in the interstitium of the kidney per total area in one field of view. The area fraction was counted using ImageJ software for 15 fields of view in each slide and presented in percent unit.

BMP-7 and HGF mRNA Expression

BMP-7 and HGF mRNA expression was assessed using RT-PCR. RNA was extracted from kidney tissue using Trizol (Invitrogen, 1559-018, Paisley) and cDNA was synthetised using ReverTra Ace (Toyobo Life Science). RT-PCR was performed to examine the expression of the following genes: BMP-7 (forward 5′-ACCCTTCATGGTGGCCTTCT-3′, reverse 5′-CCTCAGGGCCTCTTGGTTCT-3′), HGF (forward 5′-AGAAATGCAGTCAGCACCATCAAG-3′, reverse 5′GATGGCACATCCACGACCAG-3′), and GAPDH (forward 5′-ACCACAGTCCATGCCATCAC-3′, reverse 5′-TTGAGGTGGTTGTGGAAAAG-3′) was used as reference. The densitometry analysis was performed using ImageJ software.

Data Analysis

Data were presented as mean±standard error mean (SEM) and analysed using SPSS 23 software. The normality of data was assessed using Shapiro-Wilk test. If the data were normally distributed, one-way ANOVA was used, otherwise, Kruskal-Wallis test was used for statistical analysis.

RESULTS

Myofibroblast and Fibrosis Area Fractions

α-SMA immunohistochemistry showed positive expression in the interstitium of the kidney of UUO-control and UUO-chlorogenic acid groups. In SO group, α-SMA-immunopositive signal was found in the perivascular area, presenting vascular smooth muscle layers (Figure 1). The quantification results using ImageJ software showed higher mean area fraction of UUO-control groups (6.52±1.33%, U7;

18.24±1.39%, U14) compared to SO group (0.22±0.01%) and UUO-chlorogenic acid groups (1.24±0.37%, UC7; 4.58±0.61%, UC14) (Figure 2).

Shapiro-Wilk test showed that the data was not normally distributed ($p<0.05$). Data transformation was performed and still indicated that the data was not normally distributed ($p<0.05$). Therefore, statistical analysis was performed using Kruskal-Wallis test. Kruskal-Wallis test showed a significant difference among the groups ($p<0.05$). Post-hoc analysis using Mann-Whitney test showed significant differences between SO group vs other groups ($p<0.05$), U7 groups vs. UC7, U14, and UC14 groups ($p<0.05$), and U14 group vs. UUO-chlorogenic acid groups ($p<0.05$).

Sirius Red staining also showed positive labeling in the interstitium of the kidney of UUO-control and UUO-chlorogenic acid groups (Figure 1). The quantification results using ImageJ software showed higher mean area fraction of UUO-control groups (6.61±0.8%, U7; 12.98±2.31%, U14) compared to SO group (0.62±0.10%) and UUO-chlorogenic acid groups (4.76±1.03%, UC7; 3.72±0.54%, UC14) (Figure 2).

Shapiro-Wilk test showed that the data were not normally distributed ($p<0.05$). Data transformation was performed and still indicated that the data were not normally distributed ($p<0.05$). Therefore, statistical analysis was performed using Kruskal-Wallis test. Kruskal-Wallis test showed a significant difference among the groups ($p<0.05$). Post-hoc analysis using Mann-Whitney test showed significant differences between SO group vs other groups ($p<0.05$), U7 groups vs UC7, U14, and UC14 groups ($p<0.05$), and U14 group vs UUO-chlorogenic acid groups ($p<0.05$). These results indicated that chlorogenic acid administration in UUO mice model had significantly lower area fractions of α-SMA-immunopositive myofibroblasts and Sirius Red-positive collagen fibers compared to UUO-control groups.

BMP-7 and HGF mRNA Expression

The intensity of gel electrophoresis bands of BMP-7 mRNA expression showed that UUO-control groups were lower than SO and UUO-chlorogenic acid groups (Figure 3A). The densitometry analysis showed the results of SO group (2.09±0.87), U7 group (1.02±0.47), UC7 group (1.84±0.49), U14 group (1.18±0.65), and UC14 group (2.19±0.43) (Figure 3B).

Saphiro-Wilk normality test showed that the data was normally distributed ($p>0.05$). One-way ANOVA test showed that there was a significant difference among groups ($p<0.05$), while the homogeneity test suggested homogenous data between groups. The LSD post-hoc test indicated that there were significant differences between SO group vs UUO-control groups ($p<0.05$), U7 group vs UUO-chlorogenic groups ($p<0.05$), and U14 group vs UC14 group ($p<0.05$). These results indicated that BMP-7 mRNA expressions were significantly lower in UUO-control groups compared to SO group and significantly higher in UUO-chlorogenic acid group compared to UUO-control groups (Figure 3B).

The intensity of gel electrophoresis bands of HGF mRNA expression showed that UUO-control groups were lower than SO and UUO-chlorogenic acid groups (Figure 3A). The densitometry analysis showed the results of SO group (1.88±0.81), U7 group (1.06±0.31), UC7 group (1.58±0.38), U14 group (0.89±0.14), and UC14 group (1.84±0.42) (Figure 3C).

Shapiro-Wilk test showed that the data was normally distributed ($p>0.05$). One-way ANOVA test showed a significant difference among groups ($p<0.05$). The LSD post-hoc test indicated that there were significant differences between SO group vs UUO-control groups ($p<0.05$), U7 group vs UC14 group ($p<0.05$), U14 group vs UUO-chlorogenic acid groups ($p<0.05$). These results demonstrated that HGF mRNA expression were significantly higher with chlorogenic acid administration compared to UUO-control groups (Figure 3C).

DISCUSSION

UUO is a well-known mice model to induce kidney fibrosis. Kidney fibrosis is characterised by tubulointerstitial fibrosis with myofibroblast formation and structural kidney damage. Our study shows that UUO-control groups on day-7 and 14 present higher α-SMA-immunopositive and Sirius Red-positive area fractions compared to sham-operated group (Figure 1). These results are consistent with a previous study that showed progressive increase in the inflammatory cell infiltration 12 hours to 4 days after obstruction in UUO-induced kidney fibrosis model.[15] Infiltration process increases the accumulation of extracellular matrix starting on day 3.[16] There is an increase in myofibroblast formation on day-7 after the accumulation of extracellular matrix which becomes more apparent on day-14.[17] Sirius Red and PAS stainings showed that UUO model can induce interstitial fibrosis and tubular injury.[18]

Our study shows that UUO-control groups have lower BMP-7 expressions compared to sham-operated group. BMP-7 can prevent the initiation and progression of injury-induced fibrosis. BMP-7 activity has the opposite effect to the TGF-β activity (profibrosis). Interaction between BMP-7 and TGF-β begins in the early phase of kidney injury. Kidney injury induces TGF-β production and TGF-β then binds to TβRII (TGF-β receptor type II) to activate TβRI (TGF-β receptor type I). These activations carry signals by phosphorylating R-Smads (iSmads), including Smad2 and Smad3. In addition, BMP-7 binds to BMPRs (BMP receptors) to activate R-Smad1/5/8. Phosphorylated Smad forms heteromeric complex with Smad4 and the complex is then transported into the nucleus to regulate gene transcription. Heteromeric complex of BMP-7 prevents kidney fibrosis, whereas heteromeric complex of TGF-β initiates kidney fibrosis.[19] Chlorogenic acid as an anti-inflammatory substance could inhibit TGF-β.[13] Our study demonstrates that chlorogenic acid administration increases BMP-7 expression in UUO-induced kidney fibrosis mice model.

Our study also shows that UUO-control groups have lower HGF mRNA expressions compared to sham-operated group. However, there is no signicant difference between UUO-control and UUO-chlorogenic acid groups on day-7. This occurs due to compensatory mechanism in kidney tubular cells to prevent cellular death in the early phase of kidney

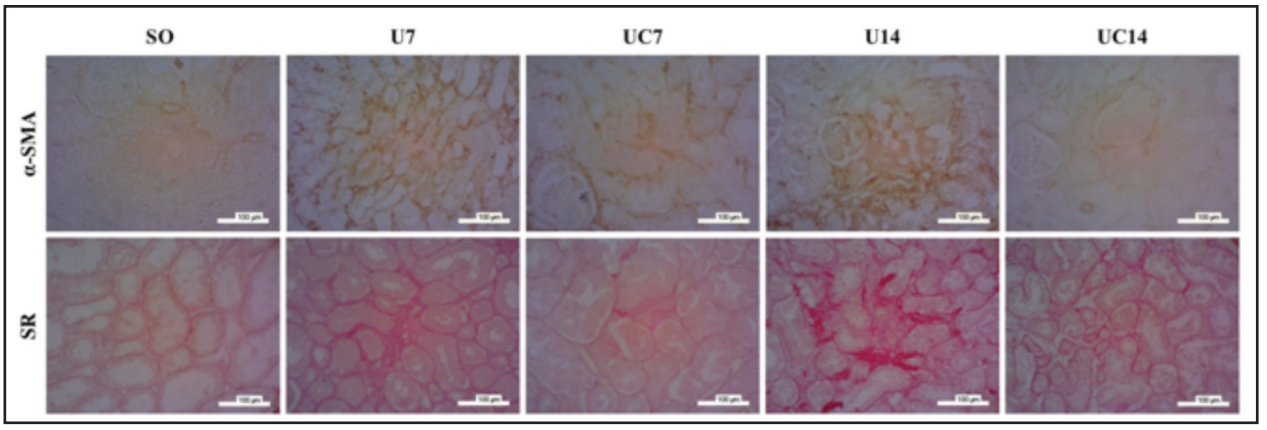

Fig. 1: Light micrographs at 400X magnification of α-SMA immunoexpression (upper) and Sirius Red-stained collagen fibers (lower) in the interstitium of the kidney of SO, UUO-control, and UUO-chlorogenic acid groups. Scale bars =100μm.

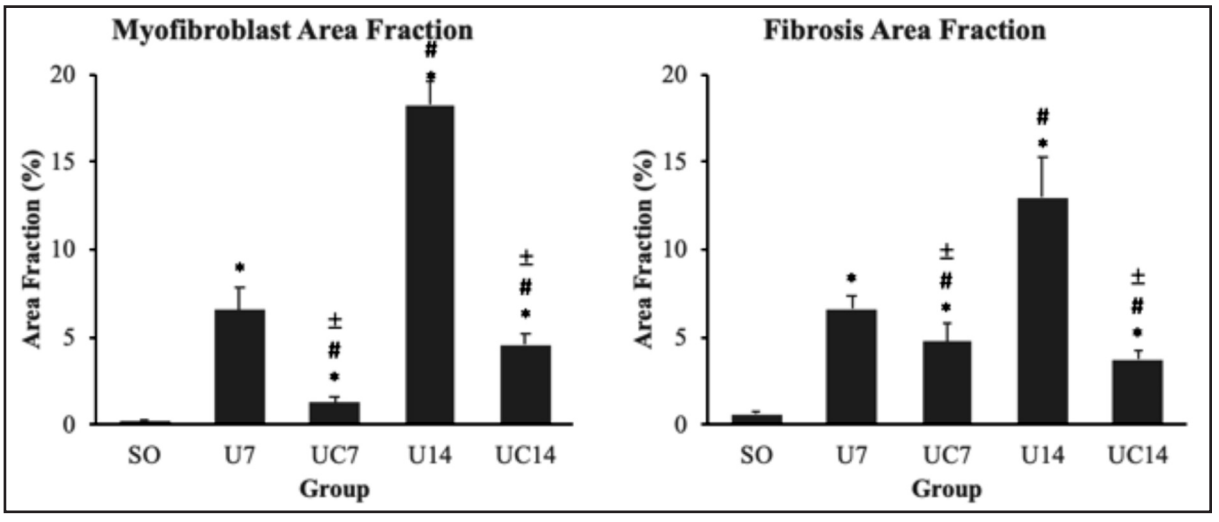

Fig. 2: Quantitative analysis on α-SMA-immunopositive (left) and Sirius Red-positive area fractions (right) in the SO, UUO-control, and UUO-chlorogenic acid groups. *)vs. SO (p<0.05), #)vs. U7 (p<0.05), ±)vs. U14 (p<0.05).

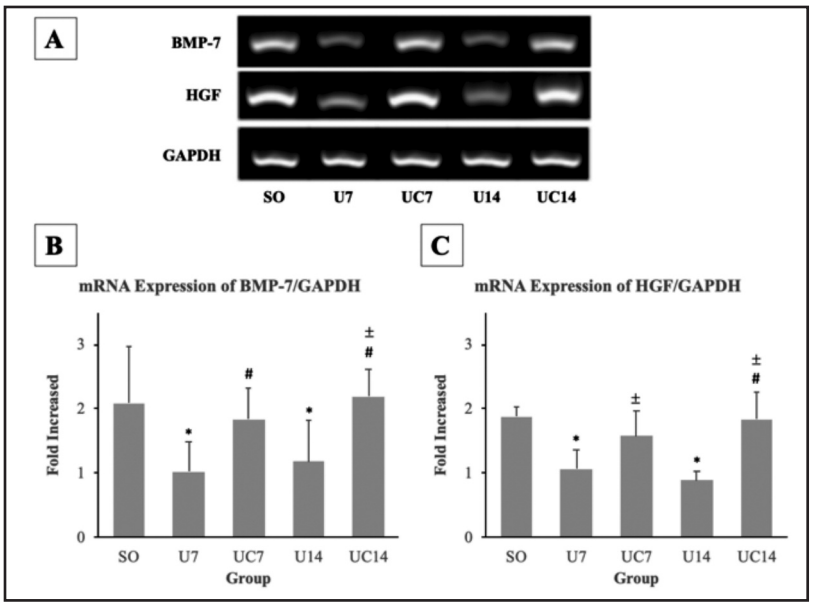

Fig. 3: A. Gel electrophoresis bands of BMP-7, HGF, and GAPDH; B. Densitometry analysis of BMP-7/GAPDH mRNA expression; C. Densitometry analysis of HGF/GAPDH mRNA expression. *)vs. SO (p<0.05), #)vs. U7 (p<0.05), ±)vs. U14 (p<0.05).

injury.[20] There is higher HGF expression in UUO-chlorogenic acid group compared to UUO-control group on day 14. Mitogenic effect of HGF could also be seen in kidney endothelial cells.[21] Kidney cells have c-Met receptor which acts as HGF receptor.[22] HGF binds to c-Met receptor and then induces autophosphorylation of the tyrosine kinase domain. This process initiates intracellular signal transduction, such as p85/p110 subunit from phosphoinositide 3-kinase (PI3K), Grb2/Sos/Ras complex, phospholypase C-γ (PLC-γ), and Gab-1 multiadaptor protein, which regulates cell regeneration, proliferation, migration, and tubulogenesis.[9] Cellular mechanism of HGF occurs in most kidney cells because c-Met expression is dispersed over most parts of the kidney.[23,24] Cellular mechanism of HGF via Smad2 and Smad3 in TGF-β1 provides inhibitory effect on podocytes apoptosis, endothelial cells apoptosis, expansion of mesangial matrix in glomerulus, while produces inhibitory effect on tubular epithelial cells apoptosis, epithelial-mesenchymal transition, interstitial fibroblast activation, and endothelial cell apoptosis in tubulointerstitium.[9]

CONCLUSION

Our study shows that chlorogenic acid administration may prevent kidney fibrosis indicated by lower area fractions of α-SMA-immunopositive myofibroblasts and Sirius Red-positive collagen fibers as well as increased antifibrotic markers, such as BMP-7 and HGF. However, further study is needed to determine the effect of chlorogenic acid on the Smad1/5/8, BMPR, and c-Met receptor regulation as an antifibrotic pathway.

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