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Full Paper

TJ-17 (Goreisan) mitigates renal fibrosis in a mouse model of folic acid-induced chronic kidney disease

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ABSTRACT

Background and purpose: TJ-17 (Goreisan), a traditional Japanese Kampo medicine, has been generally used to treat edema, such as heart failure, due to its diuretic effect. In the present study, we investigate the effects of TJ-17 on chronic kidney disease (CKD).
Methods: We the preventive action of TJ-17 against acute kidney injury (AKI) transition to CKD in vivo using a folic acid (FA)-induced mouse model. Mice were treated with food containing TJ-17 at 48 h after FA intraperitoneal injection (AKI phase).
Results: Histological analysis, as well as renal function and renal injury markers, deteriorated in mice with FA-induced CKD and were ameliorated by TJ-17 treatment. Increased levels of inflammatory cytokines and macrophage infiltration were also alleviated in mice treated with TJ-17. Renal fibrosis, a crucial factor in CKD, was induced by FA administration and inhibited by TJ-17 treatment. Pretreatment with TJ-17 did not exert an inhibitory effect on FA-induced AKI. The increase in urinary volume in FA-induced CKD mice was ameliorated by TJ-17 treatment, with a concurrent correction of reduced aquaporins expression in the kidney.
Conclusion: TJ-17 may have a novel preventive effect against inflammation, oxidative stress, and fibrosis, contributing to innovation in the treatment of CKD.

1. Introduction

The number of patients with chronic kidney disease (CKD) has recently increased and has become a crucial problem worldwide. Recently, CKD was shown to be tightly interconnected with acute kidney injury (AKI), characterized by a rapid decline in glomerular filtration.[1] AKI is caused by numerous risk factors, such as ischemia, sepsis, drug toxicity, and trauma,[2] leading to the risk of developing CKD once the patients have recovered from AKI. In addition, patients with recurrent AKI or long-term renal dysfunction are at high risk of developing CKD.[3,4] However, currently, there are no effective treatments for AKI or CKD.

Kampo medicine (Kampo) has been developed as a traditional Japanese herbal medicine derived from ancient Chinese medicine, and has been refined as a personalized medicine adapted to the health of the Japanese people for years. Kampo is prescribed as a natural herb formula according to symptom-based diagnosis[5]; therefore, scientific evidence is lacking.

The potential efficacy of traditional herbal medicines, including Kampo, against kidney disease has been reported in several small-scale clinical studies. Astragalus root (Ougi) supplementation exerts the improvement of estimated glomerular filtration rate in patients with mild to moderate CKD.[6] Yozinkodakuto significantly suppresses the elevation of serum creatinine levels in patients with CKD.[7] In experimental studies, Shichimotsu-koka-To inhibits the progression of nephrosclerosis with renal dysfunction through an antioxidant, and maintained capillaries in a rat model of Thy-1

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nephritis.[8] Juzentaihoto also suppresses renal fibrosis and inflammation in a mouse model of adenine-induced CKD.[9] In contrast, Rikkunshito fail to suppress renal fibrosis or renal dysfunction in a mouse model of angiotensin II-induced renal injury[10] and unilateral ureter obstruction-induced renal fibrosis.[11] Kampo may be a therapeutic option for delaying CKD progression, but this remains controversial.

Goreisan (TJ-17) is widely used for a regulation on body fluid. It decreases urine volume in relative symptoms such as thirst, oliguria, and sweating and ameliorates edema symptoms by promoting urine excretion. Water modulation by goreisan might be involved in the regulation of renal aquaporin-2 expression in 5/6 nephrectomized rats.[12] Thus, Goreisan might have a direct effect on the kidney; however, its effects on CKD remain unclear.

In the present study, we examine whether Goreisan, an ethical Kampo extract formulation, can prevent renal fibrosis in mice model with CKD induced by folic acid (FA).

2. Materials and methods

The ethical Kampo extract formulation (TJ-17; Goreisan) and 3D-HPLC-based profiles (Supplemental Figure 1) were provided by Tsumura & Co. (Tokyo, Japan). TJ-17 is composed of Alisma orientalis Juzep (*Alisma plantago-aquatica L.*), Polyporus umbellatus Fries (*Polyporus umbellatus*), Atractylodes chinensis (*Atractylodes lancea*), Poria cocos Wolf (*Wolfiporia extensa*) and Cinnamomom Cassia Presl (*Cinnamomum cas-sia L. J. Presl*). Folic acid (FA) was purchased from FUJIFILM Wako Chemical Co. Ltd. (Osaka, Japan). The following commercially available antibodies were used: anti-4-hydroxynonenal (4-HNE; MHN-100P, Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd., Shizuoka, Japan), α-smooth muscle actin (αSMA; A2547, Sigma-Aldrich Japan, Tokyo, Japan), F4/80 antibody (sc-59171), transforming growth factor-β1 (TGF- β 1; sc-146), and anti-β-actin (sc-47778) (Santa Cruz Biotechnology, Inc., Dallas, TX).

2.1. Mouse model of FA-induced CKD

C57BL/6J male mice (7–8-weeks-old, weighing 22–25 g) were purchased from Nippon CLEA (Tokyo, Japan) and had free access to food (Type NMF; Oriental Yeast, Tokyo, Japan) and water. Mice were randomly divided into the following groups: vehicle-injected group with food, FA-injected group with food, and FA-injected group with food containing TJ-17. The mice were intraperitoneally injected with FA (250 mg/kg) or vehicle. At 48 h after FA injection, mice were administered two doses of TJ-17 (1.5% or 3.0% in the powder diet, and 3.0% in the pellet food). Food intake, water intake, and urinary volume were measured for 24 h using a metabolic cage (CLEA Japan, Inc. Tokyo, Japan). Fourteen days after FA injection, the mice were sacrificed, and their blood and tissue samples were collected and used for analysis. To examine the pretreatment effect of TJ-17 on FA-induced AKI, mice were fed food containing TJ-17 3 days before FA injection and sacrificed 48 h after FA injection. The experimental protocol was based on previous studies of ethical Kampo extract formulations.[13] All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of the Tokushima University Graduate School, and the protocol was approved by the Institutional Review Board of the Tokushima University Graduate School (permit numbers: T2021-75 (2021/10/13) and T2022-100 (2023/1/11)).

2.2. RNA extraction and mRNA expression

RNA extraction, cDNA synthesis, and quantitative RT-PCR were performed using commercially available reagents. The procedure was performed as previously described.[14] Primer sets used in this study are listed in Table 1.

2.3. Protein extraction and western blot analysis

Protein preparation and western blotting were performed as previously described.[14] Tissue samples were homogenized or sonicated in protein lysis buffer (RIPA buffer) containing proteinase and phosphatase inhibitors, and proteins were extracted. The extracted proteins were boiled for 5 min in the Laemmli sample buffer and used for western blotting. The detected immunoreactive bands were quantified by densitometric analysis using ImageJ 1.53 software. Software (https://imagej.nih.gov/ij/).

2.4. Measurement of plasma creatinine levels

Serum creatinine levels were determined using an alkaline picrate–based assay (LabAssay Creatinine Kit, FUJIFILM Wako Chemical Co., Ltd.) as described previously.[15]

2.5. Histological analysis

Renal tubular damage was evaluated as described previously.[16] Hematoxylin and eosin (HE)-stained sections were used to score tubular injury (tubular necrosis, brush-border loss, cast formation, tubule dilatation, and tubular degeneration) as follows:0, 0–5%; 1, 6–24%; 2, 24–49%; 3, 50–74%; and 4, ≥75%.

2.6. Immunohistochemistry

F4/80 immunohistochemical staining was performed as previously described.[16] Briefly, paraffin-embedded kidney samples were cut into 2-μm sections, and deparaffinized. After blocking, the sections were incubated with primary antibody (1:500) at 4 °C overnight. Antibody distribution was visualized using a streptavidin–biotin complex assay and 3,3′-diaminobenzidine (DAB) substrate kit (Peroxidase Stain DAB Kit (Brown Stain), NACALAI TESQUE, Inc., Kyoto, Japan). To evaluate renal macrophage infiltration, 15–20 microscopic fields were randomly selected in the renal cortex and the macrophage-positive area was expressed as a percentage of the total area by using the ImageJ 1.53 software (https://imagej.nih.gov/ij/index.html).

2.7. Statistical analysis of experimental studies

Data are presented as the mean ± standard error of the mean (mean ± SEM) with dot plots. The Kruskal–Wallis test was used for comparisons between more than two groups, and the statistical significance of each difference was evaluated. Statistical significance was set at $P < 0.05$. Statistical analysis was performed using Mac Toukei kaiseki Ver.3.0 (ESUMI Co., Ltd., Tokyo, Japan).

3. Results

3.1. Effects of TJ-17 on renal histology, function, and inflammatory response in a mouse model of FA-induced CKD

We examined the protective effect of two doses of TJ-17 on FA-induced CKD mouse model. On day 2 after FA administration, the mice exhibited slightly reduced body weight, and body weight was significantly reduced in FA-treated mice although there was no significant change in kidney weight among the four groups on day 14 (Table 2). Histological analysis revealed that FA-induced kidney injury was alleviated in mice treated with food containing 1.5% or 3% TJ-17 (Fig. 1A and B). Similarly, both doses

Table 1
Sets of primer sequences.

	Forward	Reverse
Mouse lipocalin-2 (*Lcn2*)	TGGAAGAACCAAGGAGCTGT	GGTGGGGACAGAGAAGATGA
Mouse tumor necrosis factor-α (*Tnfa*)	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT
Mouse monocyte chemoattractant protein1 (*Mcp1*)	GGAGCTCATGATGTGAGCAA	GACCAGGCAAGGGAATTACA
Mouse interleukin-β (*Il1b*)	CAGGCAGGCAGTATCACTCA	TGTCCTCATCCTGGAAGGTC
Mouse collagen-1 (*Col1a1*)	GAGCGGAGAGTACTGGATCG	GTTCGGGCTGATGTACCAGT
Mouse collagen-3 (*Col3a1*)	ACCAAAAGGTGATGCTGGA	GACCTCGTGCTCCAGTTAGC
Mouse F4/80 (*Emr1*)	CTGTAACCGGATGGCAAACT	CT GTACCCACATGGCTGATG
Mouse CD68 (*Cd68*)	CTTCCCACAGGCAGCACAG	AATGATGAGAGGCAGCAAGAGG
Mouse aquaporin 1 (*Aqp1*)	CATGAAGGTGTGGACCAGTG	CTCCACCCTGGAGTTGATGT
Mouse aquaporin 2 (*Aqp2*)	TTGCCATGTCTCCTTCCTTC	TTGTGGAGAGCATTGACAGC
Mouse aquaporin 3 (*Aqp3*)	CCTCTGGACACTTGGACAT	CAACGATGGCCAGTACACAC
36B4	GCTCCAAGCAGATGCAGCA	CCGGATGTGAGGCAGCAG

Table 2
Body weight, kidney weight, renal function, food and water intake, and urinary volume in vehicle-treated mice and folic acid (FA)-treated mice with or without TJ-17.

	Control	FA	FA + TJ-17 1.5%	FA + TJ-17 3.0%
Body weight (BW) at day 0 (g)	21.9 ± 0.3	21.8 ± 0.4	22.6 ± 0.3	21.5 ± 0.5
BW at day 2 (g)	21.1 ± 0.1	20.1 ± 0.9	21.5 ± 0.5	19.9 ± 0.7
BW at day 7 (g)	21.4 ± 0.1	19.3 ± 1.2	22.8 ± 0.8	21.3 ± 1.0
BW at day 14 (g)	24.4 ± 0.4	21.4 ± 0.7**	23.9 ± 0.7	23.1 ± 1.1
Right kidney weight at day 14 (mg)	134.6 ± 5.1	123.2 ± 5.4	143.9 ± 6.0	132.5 ± 4.0
Left kidney weight at day 14 (mg)	126.3 ± 3.7	118.2 ± 4.7	137.1 ± 3.5	127.4 ± 2.7
Creatinine at day 14 (mg/dl)	0.33 ± 0.08	0.85 ± 0.13**	0.46 ± 0.13	0.37 ± 0.08$^{\#}$
Food intake (g/day)	4.0 ± 0.2	3.5 ± 0.2	ND	3.4 ± 0.2
Water intake (ml/day)	5.6 ± 0.2	5.6 ± 0.3	ND	4.5 ± 0.2*$^{\#}$
Urinary volume (ml/day)	1.2 ± 0.1	1.9 ± 0.1*	ND	1.1 ± 0.1$^{\#\#}$

Data represent mean ± SEM; $n = 6–14$; *$P < 0.05$, **$P < 0.01$ vs. vehicle mice, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. FA mice.
ND; not done.

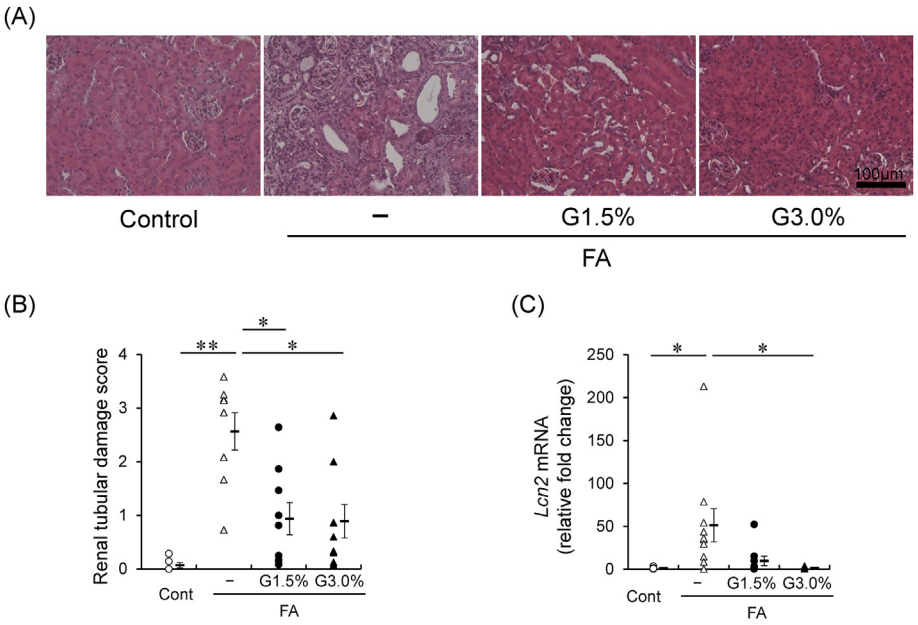

Fig. 1. TJ-17 alleviates FA-induced renal injury in mice. (A) Representative images of hematoxylin and eosin staining of the kidney sections of mice from the control group, FA-administered mice in the vehicle group, and a diet containing TJ-17 at 1.5 or 3.0% group. (B) Quantitative analysis of renal tubular damage score. Values are expressed as mean ± SEM (n = 6–9 in each group); *P < 0.05, **P < 0.01. (C) mRNA expression levels of the kidney injury marker lipocalin-2 in the kidneys of mice in all groups. Values are expressed as the mean ± SEM (n = 7–10 in each group); *P < 0.05.

of TJ-17 inhibited the worsened levels of mRNA expression of *Lcn2* as well as plasma creatinine in mice 14 days after FA administration (Fig. 1C and Table 2). The increased mRNA upregulation of inflammatory cytokines such as *Tnfa*, *Mcp1*, and *Il1b* was also diminished by TJ-17 treatment (Fig. 2). Increment of macrophage infiltration in the renal tubulointerstitium and increased *Emr1* and *Cd68* mRNA expression was observed in mice with FA-induced CKD and was inhibited by TJ-17 treatment (Fig. 3A–C). On the other hand, there were no differences in renal damage evaluated by histology, renal function, and mRNA

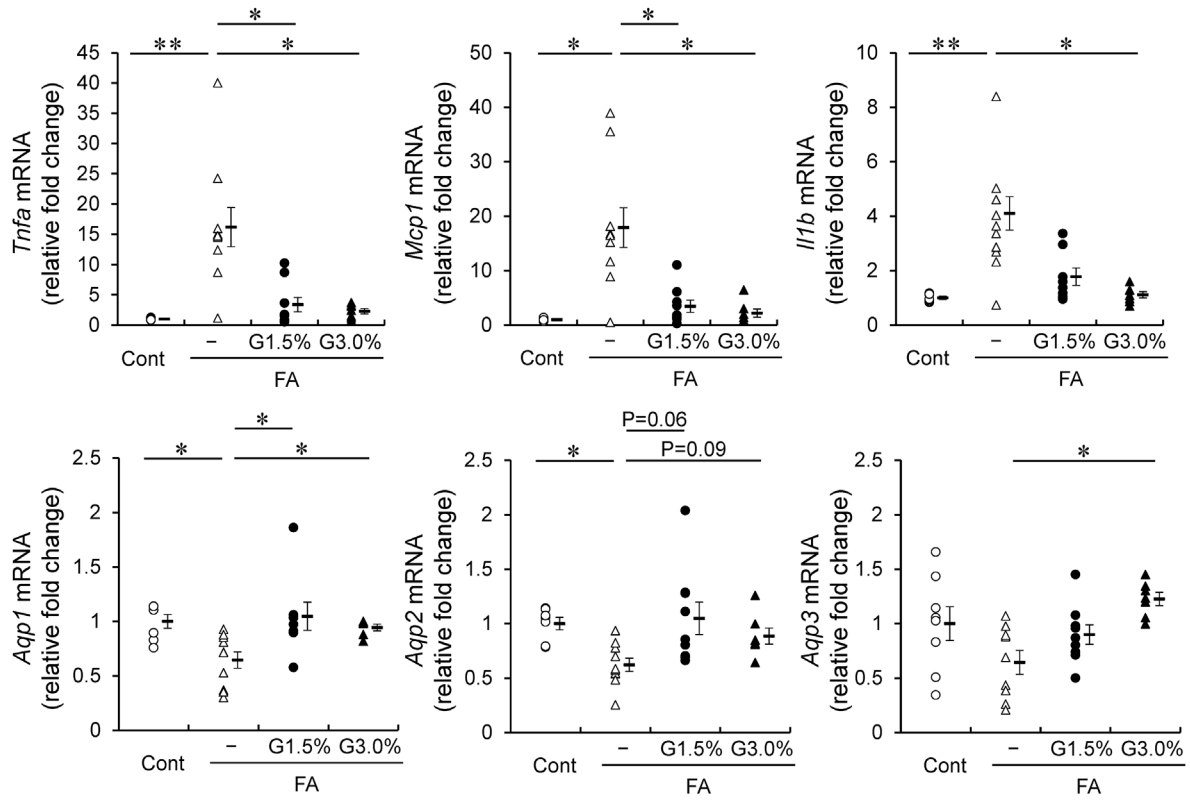

Fig. 2. TJ-17 prevents FA-induced renal inflammation. Quantitative analysis of mRNA expression of inflammatory cytokines and aquaporins in the kidneys of mice from all groups. Values are expressed as mean ± SEM (n = 7–10 in each group); *P < 0.05, **P < 0.01.

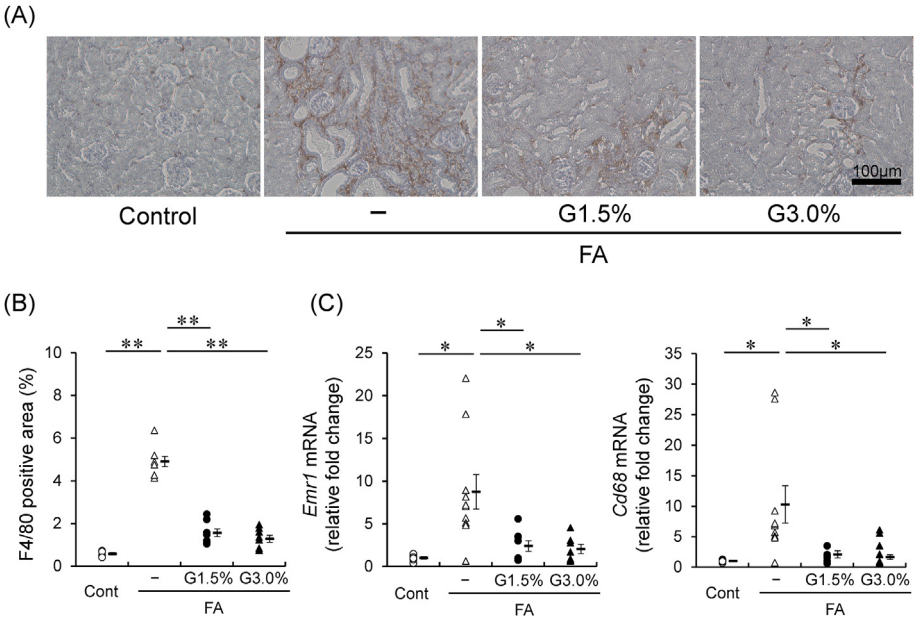

Fig. 3. Effect of TJ-17 on FA-induced renal macrophage infiltration. (A) Representative images of F4/80 immunohistochemistry in the kidneys of mice from all the groups. (B) Semi-quantitative analysis of F4/80 positive area. Values are expressed as mean ± SEM (n = 7–9 in each group); *P < 0.05. (C) Quantitative analysis of the mRNA expression of macrophage markers in the kidneys of mice from all groups. Values are expressed as mean ± SEM (n = 7–10 in each group); *P < 0.05.

expression at 48h after FA with or without TJ-17 pretreatment, indicating no protective effect on FA-induced AKI (Supplemental Figure 2). Therefore, TJ-17 might exert a protective effect against the development of CKD, but not the onset of AKI.

3.2. Effects of TJ-17 on renal fibrosis in mice with FA-induced CKD

Histological analysis showed that renal fibrotic changes were exacerbated at day 14 after FA administration, which was alleviated

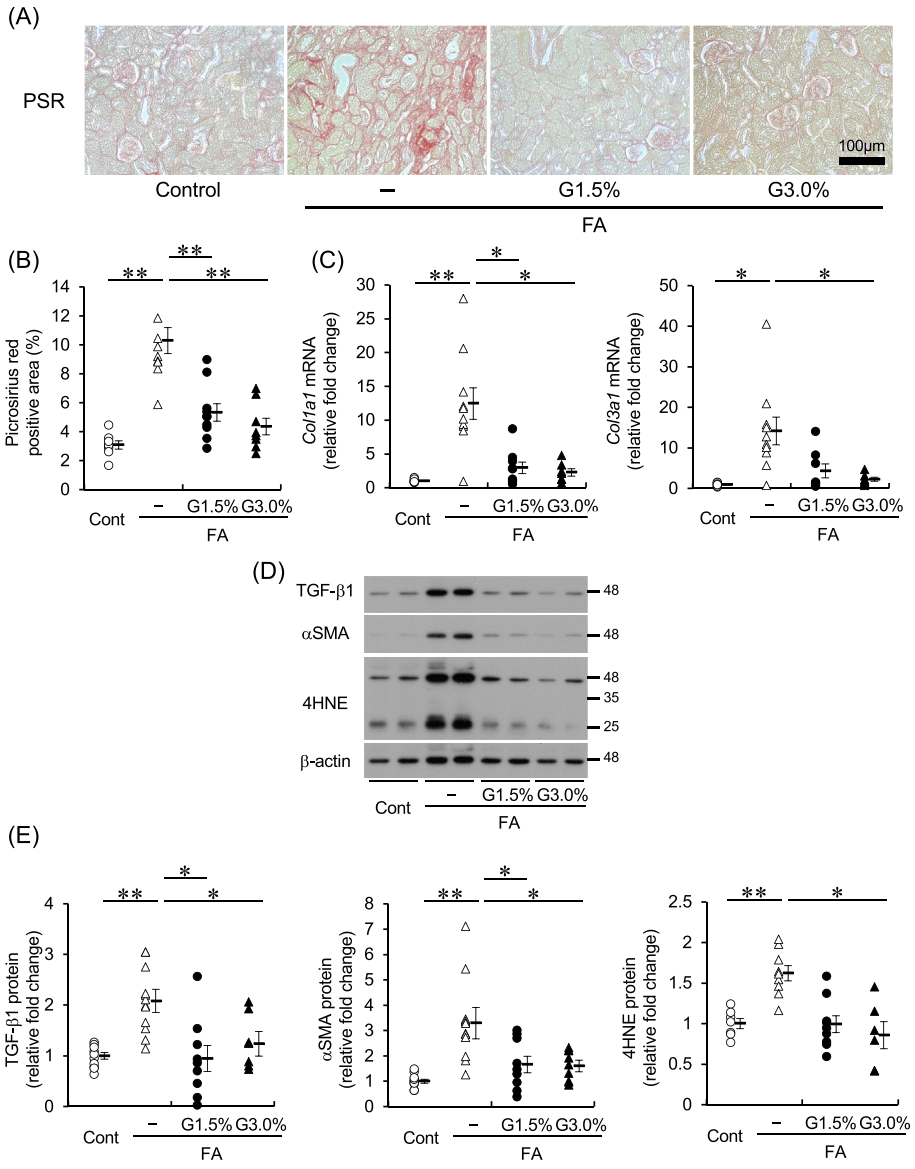

Fig. 4. J-17 alleviated FA-induced renal fibrosis. (A) Representative images of picrosirius red (PSR) staining in the kidneys of mice from all groups. (B) Semi-quantitative analysis of the PSR-positive area. Values are expressed as mean ± SEM (n = 8–10 in each group); *P < 0.05, **P < 0.01. (C) Quantitative analysis of the mRNA expression of fibrosis markers in the kidneys of mice in all groups. Values are expressed as the mean ± SEM (n = 7–10 in each group); *P < 0.05, **P < 0.01. (D) Left panel: Representative protein bands of transforming growth factor (TGF)- β 1, α smooth muscle actin (SMA), 4-hydroxynonenal (HNE), and β-actin in mouse kidneys. Right panel: Semi-quantitative densitometry analysis of TGF-β1, αSMA, 4-HNE and corrected by β-actin. Values are expressed as mean ± SEM (n = 6–9 in each group); *P < 0.05, **P < 0.01.

by treatment with both doses of TJ-17 (Fig. 4A and B). Similarly, TJ-17 suppressed the increase in mRNA expression of *Col1a1* and *Col3a1* on day 14 after FA administration (Fig. 4C). The fibrosis-related pathway (TGF-β1), as well as fibroblast (αSMA) were also activated in the kidney of mice with FA-induced CKD, and they were inhibited by TJ-17 treatment (Fig. 4D and E).

3.3. Effects of TJ-17 on oxidative stress in mice with FA-induced CKD

FA administration augmented renal lipid peroxidation (4-HNE), which was inhibited by TJ-17 (Fig. 4D and E). Collectively, the protective action of TJ-17 against CKD progression involves inhibition of inflammatory responses, oxidative stress, and fibrotic changes.

3.4. Effects of TJ-17 on daily food and water intake, and urinary volume

There was no difference in the daily food intake among control mice, FA-induced CKD mice, and FA-induced CKD mice treated with 3% TJ-17. Daily water intake was significantly diminished in FA-induced CKD mice treated with 3% TJ-17 compared to that in control and FA-induced CKD mice. Urinary volume increased in FA-induced CKD mice, which was ameliorated by TJ-17 (Table 2).

3.5. Effects of TJ-17 on aquaporins expression in the kidney

TJ-17 regulates fluid homeostasis by regulating aquaporins (AQPs) in kidney,[12] colon,[17] and brain.[18] Therefore, we examined renal AQPs expression. *Aqp1*, *Aqp2*, and *Aqp3* expression was

reduced, and they were reversed by TJ-17 in mice with FA-induced CKD (Fig. 2).

4. Discussion

We found that TJ-17 mitigated the development of renal fibrosis after the onset of AKI through the suppression of inflammation, oxidative stress, and fibrosis, although pretreatment with it did not suppress AKI. Consistently, TJ-17 alleviated FA-induced renal insufficiency, as evaluated by various markers, such as histology, renal function, Lcn2 mRNA expression. Thus, our results indicate that TJ-17 can be used as a preventive medicine against CKD.

An animal model of kidney disease induced by FA has been shown to be simple and useful for investigating AKI, CKD, and the AKI to CKD transition, mostly recapitulating the human kidney disease phenotype.[19] A single injection of high-dose FA induces AKI within 72 h, and it will consequently develop CKD if left untreated. In the present study, FA-induced CKD progression was alleviated by TJ-17 treatment after AKI onset. In contrast, TJ-17 pre-treatment failed to inhibit FA-induced AKI. In clinical settings, it is generally difficult to predict the onset of AKI, and emerging therapeutic targets for AKI are mainly aimed at improving its outcomes. Surviving patients with AKI often experience unrecovered full renal function and transition to CKD. Therefore, the inhibitory effect of TJ-17 on CKD progression is plausible, although TJ-17 does not prevent AKI.

Fibrosis is defined as the accumulation of the extracellular matrix (ECM), and its excess deposition affects tissue remodeling and dysfunction in various organs during maladaptive repair.[20] Renal fibrosis is a subsequent healing response after insufficient kidney repair due to ongoing tissue injury and inflammation, and is recognized as the common final pathway in CKD.[21] TGF-β1 is a profibrogenic cytokine signal that plays a pivotal role in renal fibrosis. TGF-β1 is one of the most important ECM regulators, both as a potent inducer of ECM synthesis and as an inhibitor of the degradation of ECM components, resulting in excess accumulation of ECM.[22] TGF-β1 also activates fibroblast-to-myofibroblast differentiation, as evaluated by αSMA upregulation,[23] thereby promoting renal interstitial fibrosis. In the present study, mice administered FA developed renal fibrosis with increased TGF-β1 and αSMA expression, which was alleviated by TJ-17 treatment, suggesting a potent efficacy of TJ-17 in delaying CKD progression.

Chronic inflammation and oxidative stress are the common causes of renal fibrosis.[24] Moreover, inflammation and oxidative stress are linked and coordinate to amplify each other,[25] further exacerbating renal fibrosis. Oxidative stress induces inflammation through NF-kβ activation and subsequent production of inflammatory cytokines.[26,27] Inflammation also increases the levels of reactive oxygen species (ROS) through NADPH oxidase 2 in a dose-dependent manner in hepatic macrophages,[28] resulting in oxidative stress. The present study showed that TJ-17 suppressed the increase in inflammatory cytokine expression and lipid peroxidation, contributing to the reduction in renal fibrosis in the kidneys of mice with FA-induced CKD.

Angiotensin II plays a pivotal role in the pathogenesis of renal diseases via inflammation and oxidative stress.[29] TJ-17 induces natriuresis by inhibiting the renin-angiotensin-aldosterone system in normal rats.[30] He et al. showed that TJ-17 treatment inhibited the increase in renal angiotensin II content in a rat model of adriamycin-induced nephrotic syndrome.[31] Therefore, we speculated that the preventive effect of TJ-17 on inflammation and oxidative stress might be partly mediated through angiotensin II suppression.

Macrophages play key roles in renal fibrosis. They are classically divided into polarizations to differentiate either the pro-inflammatory phenotype (M1: classically activated) or anti-inflammatory phenotype (M2: alternatively activated).[32] Kidney injury triggers the recruitment of circulating monocytes into the interstitial compartments in an MCP-1-denepndent manner,[33] and they differentiate into M1 or M2 macrophages depending on the local tissue environment. M1 macrophages with a pro-inflammatory phenotype secrete inflammatory mediators, such as TNF-α and ROS, which induce tissue inflammation and subsequent kidney fibrosis. M2 macrophages with an anti-inflammatory phenotype secrete anti-inflammatory mediators, such as TGF- β; however, TGF- β suppresses kidney inflammation and promotes kidney fibrosis.[34] Therefore, an increase in macrophage infiltration might be one of the factors that promote renal fibrosis during CKD progression. We found that TJ-17 diminished the FA-induced renal infiltration of macrophages, contributing to the reduction of fibrosis. As mentioned above, MCP-1 is a key chemokine that regulates the migration and infiltration of monocytes/macrophages.[35] MCP-1 is produced by many cell types, including interstitial and tubular cells and macrophages in CKD.[33] Blocking MCP1 ameliorated renal fibrosis and macrophage infiltration in mice with unilateral ureter obstruction,[36] indicating that MCP-1 intervention coordinated the injured kidney and infiltrated macrophages. In the present study, TJ-17 inhibited mRNA upregulation of *Mcp1* in FA-induced CKD, contributing to the reduction of renal macrophage infiltration.

Mice with FA-induced CKD showed a higher urine volume than control mice, although there was no difference in water intake between them. TJ-17 treatment ameliorated the increase in urine volume in FA-induced CKD mice with a concurrent reduction in water intake. AQPs dysfunction causes water-balance disorders,[37] resulting in high urine volume and low urine osmolality. A previous study showed that TJ-17 reversed the decrease in urinary osmotic pressure by reducing AQP2 and increasing AQP3 expression, with no change in AQP1 expression.[12] In contrast, our study showed that *Aqp1*, *Aqp2*, and *Aqp3* expression was reduced, which was reversed by TJ-17 in mice with FA-induced CKD, indicating the promotion of water reabsorption and reduction of urine volume. Promoting water reabsorption may lead to reduced water intake in FA-induced CKD mice treated with TJ-17. Thus, TJ-17 affects the body fluid balance in CKD mice by regulating both water intake and urine volume, which may be related to its renoprotective effect. Further investigation is required to clarify this issue.

Recently, a randomized clinical trial has started to evaluate the efficacy and safety of TJ-17 as a new congestion control strategy that add TJ-17 to the usual care in patients with heart failure (HF). Patients with HF often develop CKD, which causes a spectrum of disorders involving both the heart and kidneys, known as cardiorenal syndrome.[38] Therefore, TJ-17 might be useful in patients with cardiorenal syndrome to exert dual protective effects against both HF and CKD in the future.

5. Conclusion

The present study is the first to clarify the preventive effect of TJ-17 on CKD by inhibiting inflammation, oxidative stress, and fibrosis. This finding may contribute to the potential efficacy of Kampo medicine as a new therapeutic strategy for patients with CKD.

Author contributions

Aoi Suenaga: Investigation, Analysis. **Yasuyuki Seto:** Investigation, Analysis. **Masafumi Funamoto:** Writing - Reviewing and Editing. **Masaki Imanishi:** Writing - Reviewing and Editing. **Koichiro Tsuchiya:** Writing - Reviewing and Editing. **Yasumasa Ikeda:** Conceptualization, Methodology, Validation, Investigation, Analysis, Writing - Original draft preparation, Supervision. All data were

generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of the work, ensuring its integrity and accuracy.

Study approval

All experimental procedures involving mice were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University Graduate School, and the protocol for animal protection was approved by the Institutional Review Board of Tokushima University Graduate School (Permit Number: T30-74, T2021-75).

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2023.07.001.

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