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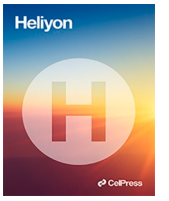

Asiaticoside ameliorates renal ischemia/reperfusion injury by promoting $CD4^+CD25^+FOXP3^+$ treg cell differentiation

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ABSTRACT

Ischemia/reperfusion injury (I/R) is the major cause of acute kidney injury, which becomes a global health problem. The effects of asiaticoside, as an anti-inflammatory drug, on renal ischemia-reperfusion injury have not been well defined.

After the $CD4^+$ cells were treated with asiaticoside, the $CD4^+$CD25+FOXP3+ Treg cell differentiation was detected by flow cytometry. The viability and release of inflammatory factors of $CD4^+$CD25+FOXP3+ Treg cell were detected by CCK-8 and ELISA. Renal I/R injury mice model was established, and the mice were pre-treated with asiaticoside or CD25 antibody or infused with Treg cells. The histological changes of renal tissue were evaluated by Hematoxylin-eosin, PAS, and Masson staining. The renal function markers were evaluated by colorimetry, the release of inflammatory factors was determined by ELISA. The Th17 and Treg cells in the blood and spleen were quantified by flow cytometry. The expressions of FOXP3 and RoR-γt in renal tissues were determined by western blotting.

Asiaticoside promoted $CD4^+$CD25+FOXP3+ Treg cell differentiation, increased the cell viability and down-regulated TNF-α, IL-1β, and IL-6, while up-regulated IL-10 of $CD4^+$CD25+FOXP3+ Treg cells. Moreover, asiaticoside ameliorated the histological damage, decreased the Th17 cells and increased Treg cells, and down-regulated the TNF-α, IL-1β, IL-6, blood urea nitrogen, serum creatinine, and RoR-γt, while up-regulated IL-10 and FOXP3 of renal I/R injury mice. Effect of asiaticoside on renal I/R injury mice was reversed by CD25 antibody whose role was further reversed by Treg cell infusing.

In conclusion, asiaticoside ameliorated renal I/R injury due to promoting $CD4^+$CD25+FOXP3+ Treg cell differentiation.

1. Introduction

Acute kidney injury (AKI) is a common clinical disease with high morbidity and mortality, and its outset leads to the development of chronic kidney disease (CKD) or even end-stage kidney disease (ESRD) [1]. Renal ischemia/reperfusion (I/R) injury is the main cause of AKI [2,3]. In various clinical practices such as surgery, shock, sepsis, trauma, and kidney transplantation, the interruption of renal blood flow lead to renal I/R which in turn causes renal tubular and endothelial cell damage, and finally cause acute kidney injury [4]. How to diagnose and treat renal I/R injury early in clinical work to improve prognosis and reduce mortality is a big challenge for

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clinicians. Evidences show that renal I/R injury is an inflammatory disease mediated by the adaptive and innate immune system [4]. To protect organs against renal I/R injury, investigators have disinterred and evaluated various therapeutic methods, including drug treatment and immunotherapy [5,6]. Recently, studies discover that regulatory T cell (Treg) plays a critical role in I/R injury, that is, Treg cells negatively regulate overmuch inflammatory reactions and might help organs protect from I/R injury [7]. Liu et al. demonstrate that Baicalin improves the pancreatic injury through actvating Treg response [6]. Studies also prove that Treg response is defective in acute kidney injury [8]; Yamamoto et al. show that Treg expansion can ameliorate renal I/R injury [9], and Luan et al. report that Resolvin D1 protects against renal I/R injury by increasing Treg percentage [10]. Therefore, finding the approach to modulate Treg response is of great significance for the treatment of renal I/R injury.

With the continuous development and extensive research of Chinese herbal medicine, many natural plants have been proven to have a good regulatory effect on the immune system, and can efficiently and stably promote, inhibit or regulate immune function in both directions, such as Baicalin, Quercetin, Zhenbao Pill, and Salidroside [11–13]. Asiaticoside (AT), a major active compounds of *Centella asiatica*, has been proved to possess multiple biological activities on different types of disease [14,15]. AT can inhibit the proliferation and chemotherapeutic drug resistance in liver cancer [16]; AT improves neonatal hypoxic-ischemic brain damage by suppressing TLR4/NF-κB/STAT3 pathway [17]; and AT induces osteogenic differentiation of human periodontal ligament cells by modulating the Wnt signaling pathway [18]. In addition, AT is recently discovered to suppress renal fibrosis development through modulating the miR-142–5p/ACTN4 axis [19]. However, the role of AT in renal I/R injury is lacking.

Therefore, in the present research, through animal and cell experiments, we investigated the effect of AT renal I/R injury and further explored whether the effect of AT on renal I/R injury is associated with the Treg cells.

2. Methods

2.1. Ethics statement

All animal testing procedures were approved by Zhejiang University Animal Care and Use Committee (approval number 202110270) on Dec 10, 2021. The ethical approval adhered to ARRIVE guidelines. All mice used in this study were fed in the specific pathogen-free condition with 12 h of light/dark cycle and a free diet. Before experiments, all mice were allowed to rear for 5 days to adapt to the environment. Efforts were made to minimize the pain of the animals.

2.2. Cell isolation, culture, and treatment

For isolation and culture of $CD4^+$ T cells [20], 8-week-old male C57BL/6 mice were firstly obtained from SiPeiFu (Beijing, China). Then, the spleens of the mice were taken out after the mice were unconscious with 5% isoflurane (R510-22, RWD, Shenzhen, China). After the spleens were ground, centrifuged, and erythrocyte lysed using BD Pharm Lyse Lysing Buffer (555,899; BD Biosciences, Franklin Lake, New Jersey, USA), the lymphocytes in the samples were stained by Pacific Blue-conjugated CD4 antibody (100,428; BioLegend, San Diego, California, USA), APC-conjugated CD62L antibody (17-0621-82; Invitrogen, Waltham, Massachusetts, USA), and PerCP-Cyanine5.5-conjugated CD44 antibody (45-0441-82; Invitrogen). Finally, the $CD4^+$ $CD44^-$ $CD62L^+$ T cells were obtained by an Attune NxT Flow cytometry (Thermo, Waltham, Massachusetts, USA). The $CD4^+$ T cells were cultured in RPMI-1640 medium (12,633,012; Procell, Wuhan, China) in the presence of 2 μg/mL CD3 antibody (16-0037-85; Invitrogen) and CD28 antibody (e16-0289-81; Invitrogen) for later use.

For isolation of $CD4^+CD25^+FOXP3^+$ Treg cells [9], $CD4^+$ T cells were firstly treated with 10 nmol AT for 24 h [21]. Then, the treated $CD4^+$ T cells were stained with PerCP-eFluor 710-conjugated CD4 antibody (46-0042-82; Invitrogen), Alexa Fluor 488-conjugated CD25 antibody (53-0251-82; Invitrogen), PE-eFluor 610-conjugated FOXP3 antibody (61-5773-82; Invitrogen). Finally, the $CD4^+CD25^+FOXP3^+$ Treg cells were obtained by a Flow cytometry. The obtained $CD4^+CD25^+FOXP3^+$ Treg cells were further treated with 100 nmol AT for 24 h, 48 h, and 72 h for use in CCK-8 assays.

2.3. Cell viability detection

Cell viability of $CD4^+CD25^+FOXP3^+$ Treg cells after being treated with 100 nmol AT for 24 h, 48 h, and 72 h was detected using CCK-8 assay. Briefly, after treatment, 10 μl CCK-8 buffer (C0037, Beyotime, Shanghai, China) were added into the cells and cultured for 3 h. Finally, the optical density (OD) value of cells was read under a Varioskan LUX Microplate reader (Thermo) at 450 nm wavelength.

2.4. Renal I/R injury model establishment

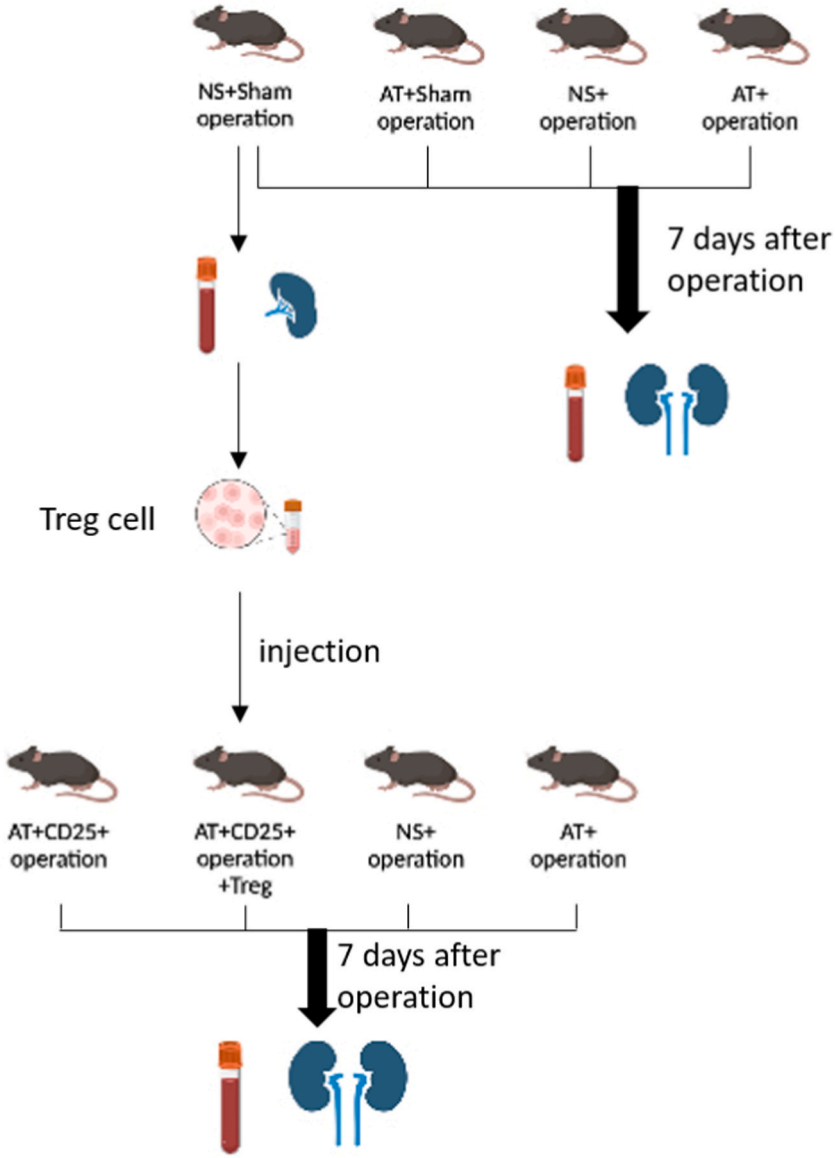
The renal I/R injury model was established according to the previous research [9]. In brief, male C57BL/6 mice with 7–8 weeks old (SiPeiFu) were unconscious with 5% isoflurane and placed a heating pad which was maintained at 35 °C during operation. Then, the renal arteries and veins were occluded with a blood vessel clip to maintain the left kidney ischemia for 30 min. After 30 min of left renal ischemia, remove the vascular clip to allow reperfusion of the left kidney. Then, the model mice were used in later experiments.

2.5. Animal grouping and treatment

Total 80 male C57BL/6 mice were involved in animal experiments, and there were two parts of animal experiments.

For the first part, 40 mice were divided into four groups which were the control group (n = 10), AT group (n = 10), model group (n = 10), and model + AT group (n = 10). Mice in the control group were gavaged with normal saline (ST341; Beyotime) once a day for three days, then the mice were received sham surgery. Mice in the AT group were gavaged with 40 mg/kg of AT once a day for three days, then the mice were received sham surgery. Mice in the model group were gavaged with normal saline once a day for three days, then the mice were received I/R injury operation. Mice in the model + AT group were gavaged with 40 mg/kg of AT once a day for three days, then the mice were received an I/R injury operation. Seven days after the operation, blood samples were obtained from the tail of all mice, then the left kidney and spleen were obtained after the mice were unconscious with 5% isoflurane. Finally, all mice were sacrificed by cervical dislocation under unconscious.

For the second part, 40 mice were divided into four group which were the model group (n = 10), model + AT group (n = 10), model + AT + CD25 group (n = 10), and model + AT + CD25+Treg group (n = 10). Mice in the model group were gavaged with normal saline once a day for three days, then the mice were received an I/R injury operation. Mice in the model + AT group were gavaged with 40 mg/kg of AT once a day for three days, then the mice were received an I/R injury operation. Mice in the model + AT + CD25 group were received I/R injury operation, besides, the mice were received intragastric gavage of 40 mg/kg AT three days before surgery for three consecutive days (once a day), and intraperitoneal injection of CD25 antibody (300 μg/mouse) one day before surgery for 2 consecutive days (once a day). Mice in the model + AT + CD25+Treg group were received I/R injury operation; besides, the mice were received intragastric gavage of 40 mg/kg AT three days before surgery for three consecutive days (once a day), and intraperitoneal injection of CD25 antibody (300 μg/mouse) one day before surgery for 2 consecutive days (once a day); in addition, the mice after operation were received Treg cells through tail vein injection with a dose of 10×10^6 cells/kg in 1 mL of sterile PBS (C0221A, Beyotime) [22]. Seven days after operation, blood samples were obtained from the tail of all mice, then the left kidney were obtained after the mice were unconscious with 5% isoflurane. Finally all mice were sacrificed by cervical dislocation under unconscious.



Animal Grouping

2.6. Analysis of Th17 and treg cells in mice blood and spleen

Analysis of Th17 and Treg cells was performed according to the previous research [7]. Briefly, the lymphocytes in spleens and blood of the mice were firstly isolated using a lymphocyte isolation medium (P8860 for spleens, P8620 for blood; Solarbio, Beijing, China). After the lymphocytes were washed with PBS, 1×10^6/mL lymphocyte suspensions were stained with CD4 antibody (46-0042-82; Invitrogen), IL-17 antibody (or Th17 cells; 11-7177-81; Invitrogen), and FOXP3 antibody (for Treg cells; 61-5773-82; Invitrogen). Finally, the Th17 and Treg cells were obtained by Flow cytometry.

2.7. Western blot analysis

Protein expressions of FOXP3 and RoR-γt in mice kidney tissues were quantified using Western blot analysis. Mice kidney tissues were lyzed by NP-40 Lysis Solution (N8032; Solarbio) which added with protease inhibitor mixture (P6730; Solarbio) and PMSF (P0100; Solarbio) to obtain the total protein in tissues. Then, the concentration of total protein was evaluated using a BCA protein assay kit (PC0020; Solarbio). After the protein was denatured by mixing with loading buffer (P1040; Solarbio) and 100 °C heating for 5 min, the protein was separated by the SDS-PAGE gel (P1200, Solarbio) and transferred onto PVDF membrane (YA1701, Solarbio).

Then, the PVDF membrane was incubated with western blocking buffer (SW3010; Solarbio) for 2 h followed by incubating FOXP3 antibody (ab215206; 1:1000, 47 kDa, Abcam, Cambridge, UK), RoR-γt antibody (ab113434; 1:2000, 55 kDa, Abcam), and GAPDH antibody (ab8245; 1:10,000, 36 kDa, Abcam) for 16 h at 4 °C. On the second day, membrane was further incubated with relative goat-anti rabbit IgG (ab6721; 1:20,000, Abcam), goat-anti mouse IgG (ab6789; 1:10,000, Abcam), or donkey-anti goat IgG (ab6885; 1:10,000, Abcam) antibody for 2 h. At last, after the membrane was added with ECL Luminescent Liquid (M41129, MERYER), the protein signaling on the membrane was examined by Image Lab 3.0 detector (Bio-Rad, Hercules, California, USA).

2.8. Hematoxylin-eosin, periodic aci-schiff reaction (PAS), and masson staining

Before histopathology staining, the mice kidney tissues were fixed with 4% tissue fixative buffer (P1110; Solarbio), embedded with paraffin (YA0012; Solarbio), cut into 5 μm slice, and incubated with xylene (B50009, MERYER, Shanghai, China). Then, the tissue was successively incubated with 100% ethanol (E809056; Macklin, Shanghai, China) for 5 min, 90% ethanol for 2 min, 80% ethanol for 2 min, 70% ethanol for 2 min, and washed under distilled water for 2 min.

For Hematoxylin-eosin staining, the staining kit (C0105S) was obtained from Beyotime. The prepared tissue was then stained with hematoxylin for 10 min followed by staining with eosin for 1 min. Subsequently, the tissue was then incubated with 70% ethanol for 10 s, 80% ethanol for 10 s, 90% ethanol for 10 s, 100% ethanol for 10 s, and xylene for 5 min in turn. At last, after being sealed with neutral gum (G8590, Solarbio), the tissues were observed by THUNDER imaging system (Leica, Weztlar, Germany) under × 100 magnification.

For PAS staining, PAS staining kit (C0142S) was also bought from Beyotime. The prepared tissue was then incubated with the periodic acid solution for 10 min. After the tissue was washed with distilled water for 5 min, hematoxylin was used to stain the tissue for 30 s. Then, the tissue was incubated with 90% ethanol for 2 min, 100% ethanol for 2 min, and xylene for 5 min in turn. At last, after being sealed with neutral gum, the tissues were observed by THUNDER imaging system under × 100 magnification.

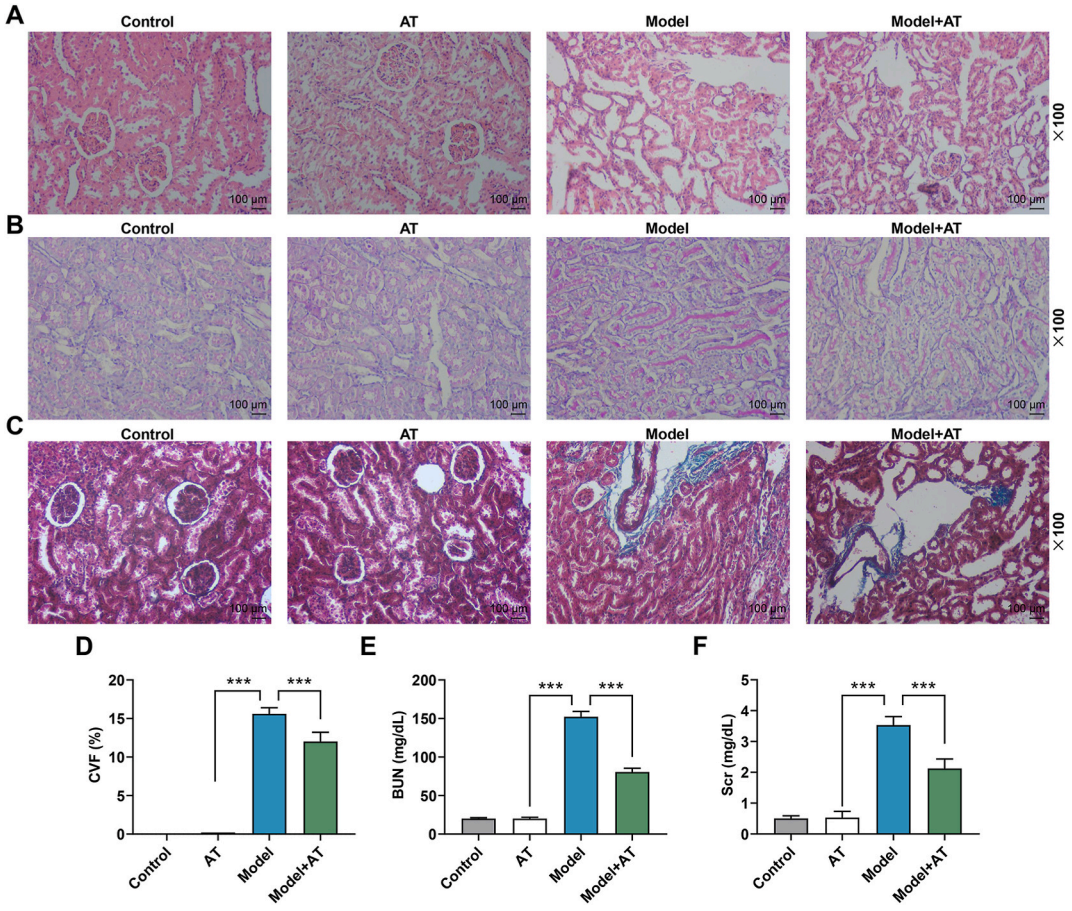


Fig. 1. AT attenuated renal histological damage and deterioration of renal function after renal I/R (A–F) After the renal I/R mice model was established and pre-treated with AT, the histological damage of renal tissue was examined through Hematoxylin-eosin staining (A; magnification × 100), PAS staining (B; magnification × 100), and Masson staining (*C*-D; magnification × 100), and the serum BUN (E) and Scr (F) were evaluated by colorimetry. ***$P < 0.001$.

For Masson staining, the staining kit (G1340) was purchased from Solarbio. The prepared tissue was then incubated with Weigert iron hematoxylin buffer for 8 min. After the tissue was incubated with acidic ethanol buffer for 10 s and washed with distilled water, Masson blue solution was used to incubate the tissue for 4 min followed by washing with distilled water for 1 min. Then, the tissue was stained by ponceau magenta staining solution for 8 min, washed by phosphomolybdic acid solution for 2 min, and stained with aniline blue staining solution for 2 min. After that, the tissue was incubated with 95% ethanol for 2 s, 100% ethanol for 10 s, and xylene for 2 min in turn. At last, after being sealed with neutral gum, the tissues were observed by THUNDER imaging system under × 100 magnification.

2.9. Renal function analysis

Renal function index including blood urea nitrogen (BUN) and serum creatinine (Scr) were analyzed as previous research [23]. BUN detection kit (C013-1-1) was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and SCr detection kit (ml037580) was obtained from mlbio (Shanghai, China). The content of BUN and SCr in mice blood was detected using a Varioskan LUX Microplate reader according to the manufacturer's instructions of BUN and SCr detection kits.

2.10. ELISA assay

The content of TNF-α, IL-1β, IL-6, and IL-10 in kidney tissues and cells supernatant was detected using ELISA assay. The mouse ELISA kits for TNF-α (ml002095), IL-1β (ml063132), IL-6 (ml002293), and IL-10 (ml037873) were purchased from mlbio. For

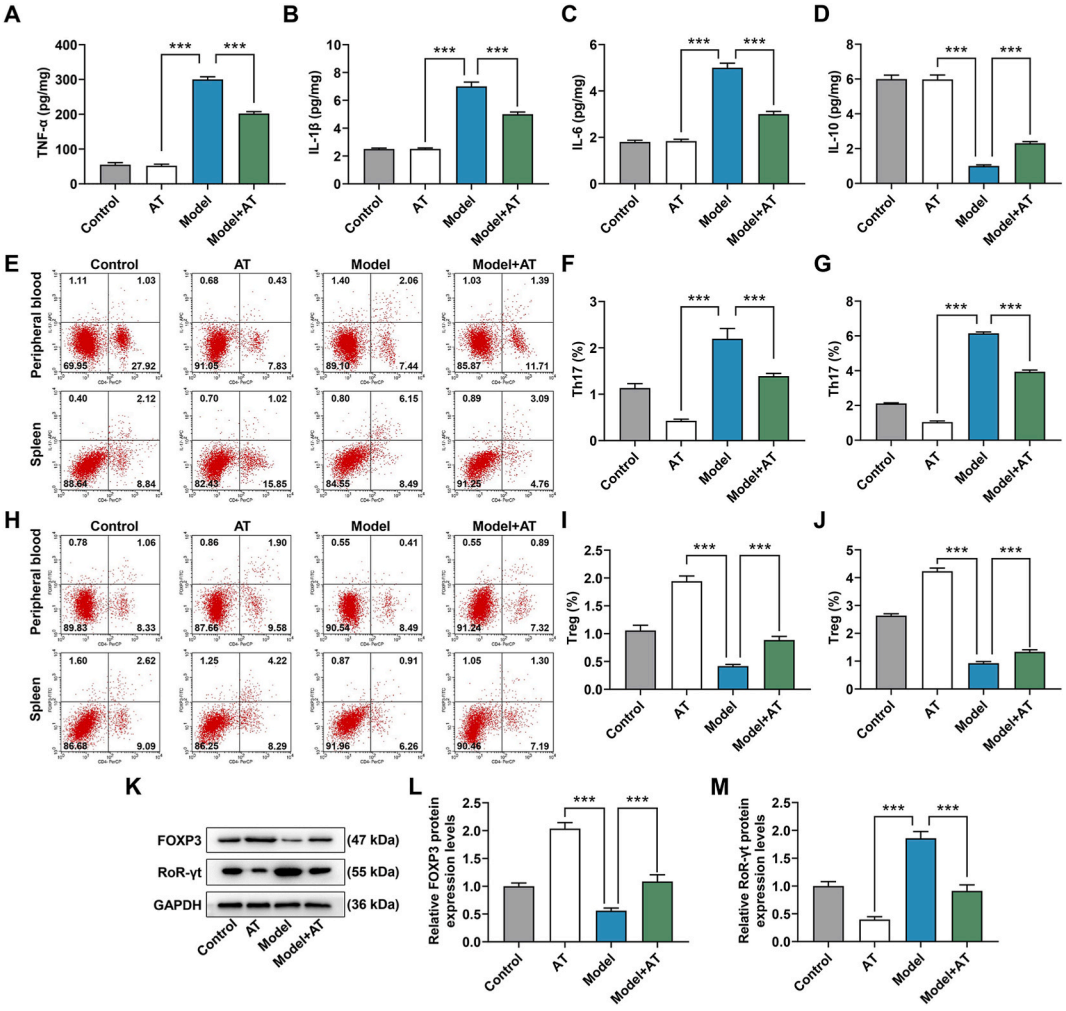


Fig. 2. AT regulated the release of inflammatory factors by modulating the Th17/Treg balance in renal I/R injury mice (A–M) After the renal I/R mice model was established and pre-treated with AT, the releases of inflammatory factors including TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D) in mice serum were detected by ELISA; the Th17 cells (*E*–G) and Treg cells (H–J) in blood and spleen were evaluated by flow cytometry; and the expressions of FOXP3 (K, L) and RoR-γt (K, M) in mice renal tissues were quantified using Western blot assays. ***$P < 0.001$.

detection, 50 μl supernatant of kidney tissues after homogenized ultrasonically and cell culture supernatant were respectively added into specific 96-well plate, then 50 μl biotin-labeled antibodies was further added into each well for reaction at 37 °C for 1 h. After washing with wash buffer, 80 μl of affinity streptin-HRP was added into each well for reaction at 37 °C for 30 min followed by washing with wash buffer. Then, 50 μl of A buffer and B buffer was further added into each well to react at 37 °C for 10 min. After 50 μl stopping buffer was added into each well, the OD value of each well was read under Varioskan LUX microplate reader at 450 nm wavelength.

2.11. Statistical analysis

All data were the mean values of 3 independent experiments and analyzed using SPSS version 22.0 software. Independent-samples T-test was used to analyze data from two groups, One-way ANOVA with Bonferroni post-hoc test was applied to analyze data from multiple groups. Statistically difference was established at a probability value of $P < 0.05$. Statistical data were presented as Mean ± SD.

3. Results

3.1. AT attenuated renal histological damage and deterioration of renal function after renal I/R

After the renal I/R model was established and pre-treated with AT, the histological of all mice were observed using Hematoxylin-eosin staining (Fig. 1A), PAS staining (Fig. 1B), and Masson staining (Fig. 1C–D). The histological morphology of renal tissue of mice in the control and AT group were normal. While, necrosis, vacuolization, cast formation, and absence of brush border in renal tubular epithelial cells were observed in mice renal tissue in the model group. After the model mice pre-treated with AT, the histological damage was attenuated by AT after 7 days. In addition, images of masson staining showed that collagen volume fraction (CVF) in the model group was significantly increased when compared with AT group ($P < 0.001$), while, the increased CVF was then decreased by AT ($P < 0.001$). AT effectively improved the pathological damage of glomeruli and renal tubules.

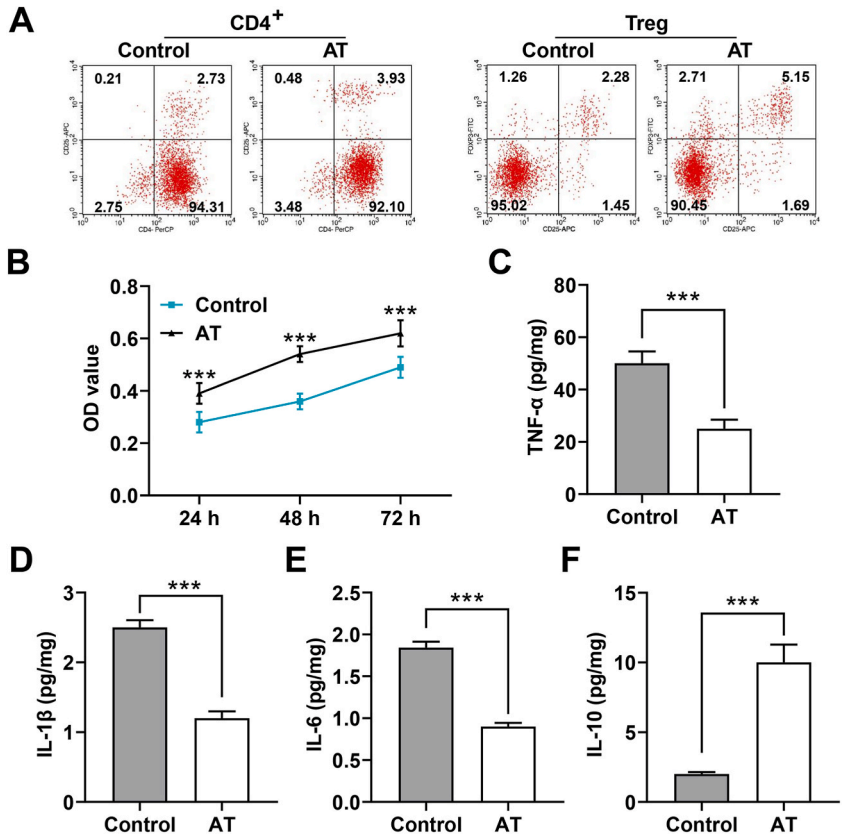


Fig. 3. AT promoted the $CD4^+CD25^+FOXP3^+$ Treg cell differentiation and increased the cell viability and regulated the releases of inflammatory factors of $CD4^+CD25^+FOXP3^+$ Treg cells (A) After the $CD4^+$ cells were treated with 100 nmol AT for 24 h, the percentage of $CD4^+CD25^+FOXP3^+$ Treg cells in total cells was evaluated by flow cytometry. (B) The viability of $CD4^+CD25^+FOXP3^+$ Treg cells after being treated with 100 nmol AT was detected by CCK-8 assays. (*C*–F) The release of the inflammatory factor of $CD4^+CD25^+FOXP3^+$ Treg cells after being treated with 100 nmol AT for 24 h was evaluated by ELISA. ***$P < 0.001$.

Furthermore, the renal function markers including serum BUN and Scr were evaluated, as illustrated in Fig. 1*E*–F. The BUN and Scr in the model group was remarkably increased ($P < 0.001$), which further decreased by AT ($P < 0.001$). And AT alone had no effect on the content of BUN and Scr. All these phenomena demonstrated that AT attenuated renal histological damage and deterioration of renal function after renal I/R.

3.2. AT regulated the release of inflammatory factors by modulating the Th17/Treg balance in renal I/R injury mice

Then, the release of inflammatory factors in renal I/R injury mice was determined through ELISA (Fig. 2A–D), as the results indicated that the releases of TNF-α, IL-1β, and IL-6 in serum of model mice were up-regulated ($P < 0.001$) and the release of IL-10 in serum of model mice was decreased ($P < 0.001$). Besides, AT then decreased the up-regulated TNF-α, IL-1β, and IL-6 and increased the down-regulated IL-10 in model mice ($P < 0.001$). Subsequently, the Th17 cells (Fig. 2*E*–G) and Treg cells (Fig. 2H–J) in blood and spleen were evaluated and the results exhibited that in the model group, the Th17 cells were increased and Treg cells were decreased

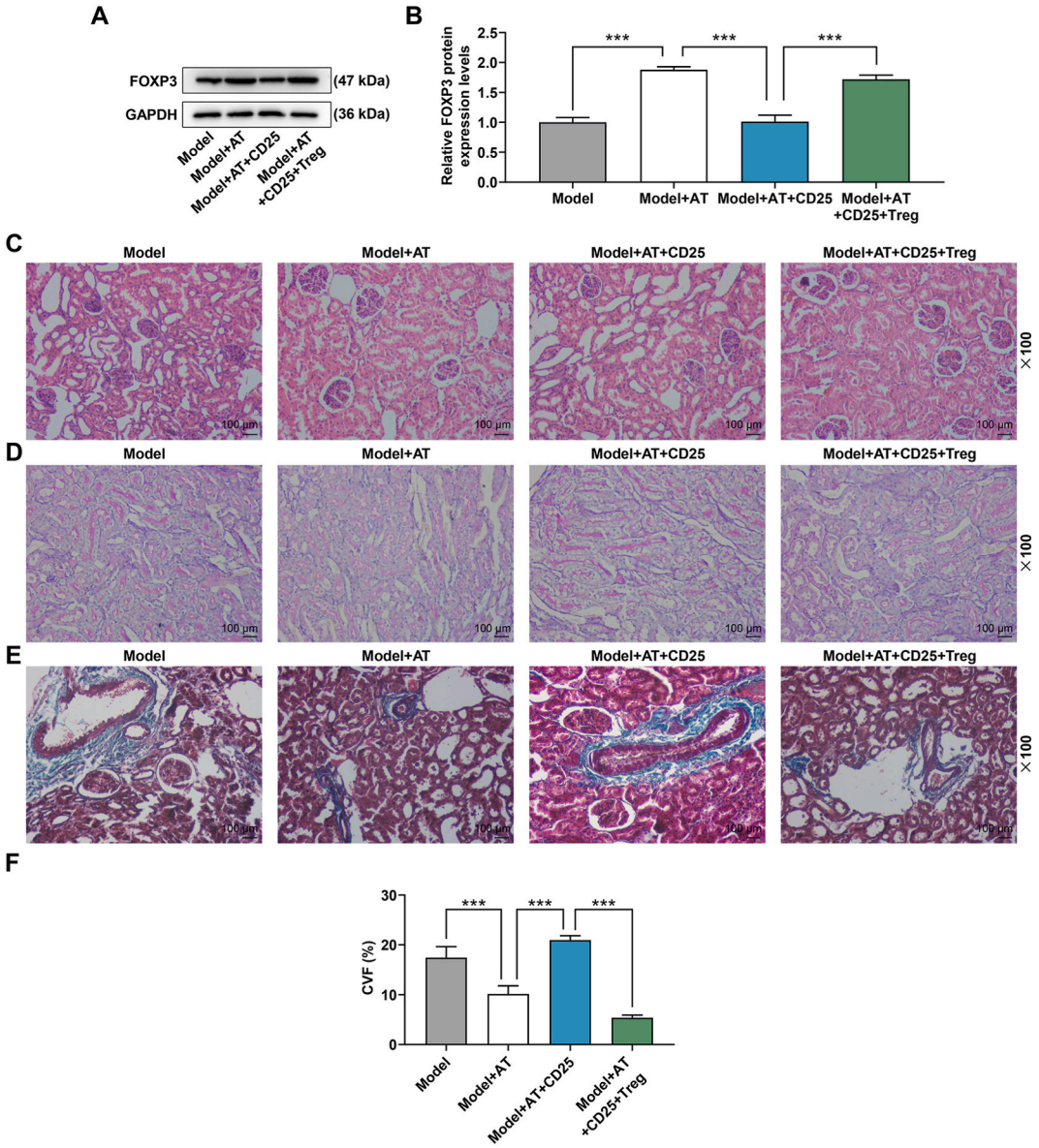


Fig. 4. AT attenuated renal histological damage of renal I/R injury mice by promoting $CD4^+CD25^+FOXP3^+$ Treg cell differentiation (A–F) After the renal I/R mice model was established, the expression of FOXP3 in renal tissue was detected by Western blot assay (A–B), the histological damage of renal tissue was examined through Hematoxylin-eosin staining (C; magnification × 100), PAS staining (D; magnification × 100), and Masson staining (*E*-F; magnification × 100). ***$P < 0.001$.

when compared with the AT group ($P < 0.001$), after AT treatment, the up-regulated Th17 cells in model mice were decreased and the down-regulated Treg cells in model mice were increased by AT ($P < 0.001$). Furthermore, the expressions of the specific transcription factor for Treg cells (FOXP3) and for Th17 cells (RoR-γt) were quantified, as illustrated in Fig. 2I-M. In the model mice, the expression of FOXP3 was decreased and RoR-γt was increased as compared to the AT group ($P < 0.001$). While, AT further up-regulated the decreased FOXP3 and down-regulated the increased RoR-γt in comparison of the model group ($P < 0.001$). This part of the discoveries indicated that AT regulated the release of inflammatory factors by modulating the Th17/Treg balance in renal I/R injury mice.

3.3. AT promoted the $CD4^+CD25^+FOXP3^+$ treg cell differentiation and increased the cell viability and regulated the releases of inflammatory factors of $CD4^+CD25^+FOXP3^+$ treg cells

To verify that AT affected $CD4^+CD25^+FOXP3^+$ Treg cells, a series in vitro assays were then carried out. As shown in Fig. 3A, after the $CD4^+$ cells were treated with AT, the percentage of $CD4^+CD25^+FOXP3^+$ Treg was significantly up-regulated ($P < 0.001$). Soon afterward, the viability of $CD4^+CD25^+FOXP3^+$ Treg cells after being further treated with AT was increased (Fig. 3B, $P < 0.001$). Besides, the releases of the inflammatory factor in the $CD4^+CD25^+FOXP3^+$ Treg cells treated with AT were evaluated (Fig. 3C–F), and the results showed that the releases of TNF-α, IL-1β, and IL-6 were down-regulated by AT ($P < 0.001$) while the release of IL-10 was up-regulated by AT ($P < 0.001$). These discoveries made us conjecture that the role of AT on renal I/R injury mice was realized by modulating $CD4^+CD25^+FOXP3^+$ Treg.

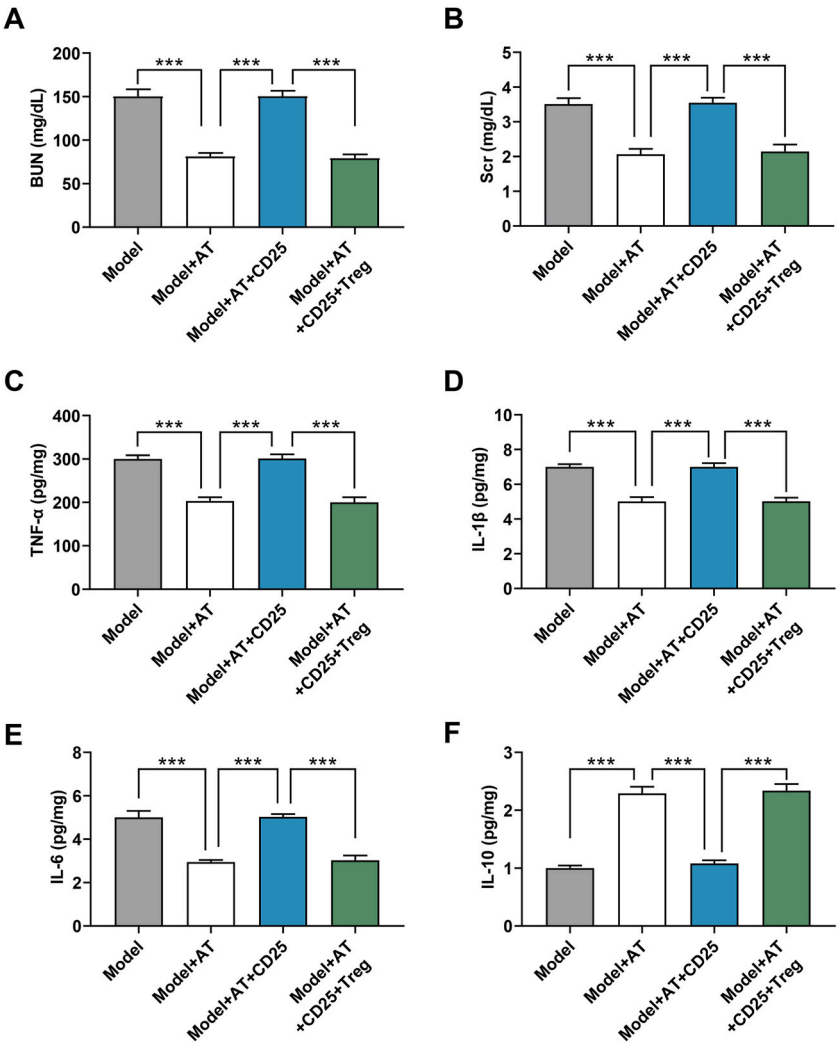


Fig. 5. AT attenuated deterioration of renal function, and inflammation of renal I/R injury mice by promoting $CD4^+CD25^+FOXP3^+$ Treg cell differentiation (A–K) After the renal I/R mice model was established, the serum BUN (A) and Scr (B) were evaluated by colorimetry, the releases of inflammatory factors including TNF-α (C), IL-1β (D), IL-6 (E), and IL-10 (F) in mice serum were detected by ELISA. ***$P < 0.001$.

3.4. AT attenuated renal histological damage, deterioration of renal function, and inflammation of renal I/R injury mice by promoting $CD4^+CD25^+FOXP3^+$ treg cell differentiation

To further demonstrate that the improving effect of AT on renal I/R injury mice was realized by modulating $CD4^+CD25^+FOXP3^+$ Treg cell differentiation, the renal I/R injury mice model was established again and the mice were pre-treated with AT and CD25 antibody, and further infused Treg cells. Seven days after the operation, the expressions of FOXP3 in renal tissue was firstly evaluated and we discovered that AT up-regulated the FOXP3 when compared with the model group (Fig. 4A–B; $P < 0.001$), while CD25 antibody decreased the FOXP3 expression as compared to the model + AT group (Fig. 4A–B; $P < 0.001$), besides, infusing Treg cells further reversed the effect of CD25 antibody (Fig. 4A–B; $P < 0.001$). Histological morphology of mice renal tissue was then observed. As exhibited in Fig. 4C, necrosis, vacuolization, cast formation, and absence of brush border in renal tubular epithelial cells of mice in the model group which further improved by AT. The similar histological morphology changes to the model group was discovered in the model + AT + CD25 group, and the histological morphology damage in model + AT + CD25 group was then improved by Treg cells infusing. We found the similar tissue changes from PAS and Masson staning (Fig. 4D–E). Meanwhile, CVF was analyzed based on the staining results of Masson, as illustrated in Fig. 4F, AT down-regulated the CVF when compared with the model group ($P < 0.001$), while CD25 antibody increased the CVF as compared to the model + AT group ($P < 0.001$). Besides, infusing Treg cells further reversed the effect of CD25 antibody ($P < 0.001$).

The renal function markers including serum BUN and Scr were further evaluated, as illustrated in Fig. 5A–B, AT down-regulated the BUN and Scr when compared with the model group ($P < 0.001$), while CD25 antibody increased the BUN and Scr as compared to the model + AT group ($P < 0.001$). Besides, infusing Treg cells further reversed the effect of CD25 antibody ($P < 0.001$). Meanwhile, the release of inflammatory factors in renal I/R injury mice renal tissue was determined through ELISA (Fig. 5C–F), as the results indicated that AT down-regulated the TNF-α, IL-1β, and IL-6 while up-regulated IL-10 when compared with the model group ($P < 0.001$), while CD25 antibody increased the TNF-α, IL-1β, and IL-6, and decreased IL-10 as compared to the model + AT group ($P < 0.001$), besides, infusing Treg cells further reversed the effect of CD25 antibody ($P < 0.001$). All these findings demonstrated that AT attenuated renal histological damage, deterioration of renal function, and inflammation of renal I/R injury mice by promoting $CD4^+CD25^+FOXP3^+$ Treg cell differentiation.

4. Discussion

AT is proved to possess the therapeutic effect, such as anti-inflammatory, anti-tumor, anti-fibrosis, neuroprotective, anti-oxidation on different types of disease [16,24,25]. However, there are few reports about the role of AT on renal I/R injury. After establishing renal I/R model mice which pre-treated with AT, we discovered that AT significantly attenuated the histological damage of renal I/R mice, and also decreased the up-regulated some markers of renal function [26]. Our findings were similar with previous research that AT alleviates cerebral I/R injury and attenuates memory impairment induced by transient cerebral I/R mice [27,28].

Renal I/R injury is an inflammatory disease [4]. Recent studies have found that as Th17/Treg cell homeostasis, which is closely linked to Th1/Th2 cell homeostasis, the imbalance of its balance is a key factor that promotes the occurrence of many autoimmune and inflammatory diseases, including renal injury [7]. Th17 is a helper T lymphocyte differentiated from Th0 cells under the stimulation of IL-6 and IL-23, and Th17 can activate neutrophils or participate in macrophage-mediated tissue damage, thereby inducing tissue inflammation [29]. Unlike Th17 cells, Treg cells negatively regulate overmuch inflammatory reactions and might help organs protect from I/R injury [30,31]. The previous study has been discovered that Th17 cells are increased while Treg cells are defective in renal injury [8], of course, TNF-α, IL-1β, and IL-6 are up-regulated while Treg cells IL-10 is down-regulated in renal I/R injury [32]. In the present research, after we discovered that AT down-regulated the increased TNF-α, IL-1β, and IL-6, while up-regulated the decreased IL-10 in renal I/R injury mice, we speculated that the role of AT in renal I/R was realized by modulating the balance of Th17 and Treg cells. Then the results of flow cytometry showed that Th17 cells in both blood and spleen were up-regulated in renal I/R injury mice which were then reversed by AT, and Treg cells were opposite with Th17 cells. Previous study found that Treg cell was not only present in the blood and spleen, they also infiltrate the kidney tissue after renal ischemia-reperfusion [33]. The effect of AT on Treg cells was mainly through directly increasing the number of Treg cells in the kidney tissue or promoting the transfer of cells outside the kidney to the kidney tissue, which remained to be discussed. Considering that FOXP3 is the specific transcription factor of Treg cells while RoR-γt is the specific transcription factor of Th17 cells [34,35], we further evaluated the expressions of FOXP3 and RoR-γt and further discovered that AT up-regulated the decreased FOXP3 while down-regulated the increased RoR-γt in renal I/R injury mice, suggesting that the attenuated role of AT was achieved by modulating the balance of Th17/Treg cells.

The effect of plant ingredients on the disease through Treg cells can be achieved by promoting the differentiation of Treg cells, exactly, is the differentiation of $CD4^+CD25^+FOXP3^+$ Treg cells [36]. Furthermore, the expansion of Treg cells had the ability to ameliorate kidney I/R injury in mice [9]. Consistently, we discovered that AT treatment promoted the differentiation of $CD4^+CD25^+FOXP3^+$ Treg cells from CD4 cells, also, AT treatment further up-regulated the cell viability and IL-10 while down-regulated the TNF-α, IL-1β, and IL-6 of $CD4^+CD25^+FOXP3^+$ Treg cells, indicating AT promoted the $CD4^+CD25^+FOXP3^+$ Treg cells differentiation to attenuate the renal I/R injury in mice, which was further demonstrated by the final data of in vivo experiments where mice were further treated with CD25 or infused with Treg cells. Wherase, we did not thoroughly investigate which key molecules of AT affect the differentiation of Treg cells in this study.

To conclude, our research proved that AT attenuated renal histological damage, deterioration of renal function, and inflammatory reactions after renal I/R injury in mice by promoting the $CD4^+CD25^+FOXP3^+$ Treg cell differentiation. Although there is still limitation such as the optimal concentration of AT to treat the renal I/R injury mice was needed more exploration, the present discovery in

our research is greatly significant to the diagnosis and treatment for renal I/R injury.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Author contribution statement

Shengjie Tang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Xiangcheng Xie: Conceived and designed the experiments.
Ming Wang: Contributed reagents, materials, analysis tools or data.
Wei Wei: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17390.

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