Life Sciences 224 (2019) 41–50

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Adefovir accumulation and nephrotoxicity in renal interstitium: Role of organic anion transporters of kidney

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ARTICLE INFO

Keywords:
Drug-drug interaction
Renal interstitium
Organic anion transporters
Drug-induced nephrotoxicity

ABSTRACT

Common characteristics of drug induced nephrotoxicity are renal tubular and interstitial injury. Many studies have only focused on renal tubular injury. However, less is known about the effects of drugs in the renal interstitium on the nephrotoxicity. The aim of this study was to investigate the pharmacokinetics of adefovir (ADV) and the nephrotoxicity in the renal interstitium. Rats were treated with ADV alone or in combination with probenecid for 1, 7, 14, or 28 days. The renal interstitial fluid was collected by renal microdialysis. The concentration of ADV was determined by HPLC-MS/MS. Nephrotoxicity was evaluated by biochemical parameters or histological analysis. The results showed that organic anion transporters (OATs) inhibitor probenecid significantly increased the area under concentration-time curves (AUC) and peak concentration (C_{max}) of ADV in the renal interstitium, while the clearance (CL) in the renal interstitium was decreased in the ADV plus probenecid group compared to the ADV groups. After long-term treatment, interstitial fibrosis was present in the ADV plus probenecid group, whereas no trace of that could be detected in the ADV groups. Furthermore, a decrease was observed in the expression of OATs/Oats, which was dependent upon the concentrations and time of ADV treatment. In conclusion, it is possible that ADV could be accumulated in the interstitium when Oats were inhibited, which could cause renal interstitial fibrosis. Simply reducing cell uptake in long-term treatment might not be an effective method to protect against chronic nephrotoxicity.

1. Introduction

Renal elimination plays a vital role in drug clearance. Among the top 200 market drugs, approximately 30% use renal clearance as the major clearance pathway [1]. Thus, the kidney is a frequent site of drug toxicity. Interestingly, although the drug elimination function of the kidney is performed by the nephrons, including the glomerulus and the renal tubules, drug induced nephrotoxicity is described most commonly as producing tubular, interstitial or, most often, combined tubulointerstitial injury, while drug-induced glomerular injury is much less frequent [2].

It is widely accepted that high concentrations of drugs or their metabolites or their accumulation in tissues can lead to organ damage, failure and even death. In the past, the physiological role of the interstitium of the kidney has received comparatively little attention, which might be partially attributed to the fact that the interstitium was mostly considered as a passive tissue that structurally supported the tubular epithelium [3]. Thus, many researchers assumed that nephrotoxicity induced by drugs could generally be attributed to the accumulation of drugs or their metabolites in tubular epithelial cells, and they investigated the reasons for the accumulation of drugs or their metabolites in proximal tubular cells. Uptake transporters located in the basolateral membrane of the tubular epithelium facilitate solutes from the blood to enter the tubular cell, while efflux transporters located in the luminal membranes of the tubular epithelium facilitate solutes to exit of epithelium (Fig. 1). Thus, transporters play a crucial role in controlling the intracellular concentrations of drugs and their metabolites [4]. Nephrotoxicity induced by drugs may be caused either by excessive cell uptake or by decreased efflux from the proximal tubular cell, and reduced cellular uptake could protect against nephrotoxicity and decrease renal clearance [5].

However, until now, little was known about the effects of drugs in the renal interstitium on renal interstitial injuries. Besides structurally supported the tubular epithelium, the renal interstitium plays a role in

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https://doi.org/10.1016/j.lfs.2019.03.042
Received 14 December 2018; Received in revised form 6 March 2019; Accepted 18 March 2019
Available online 19 March 2019

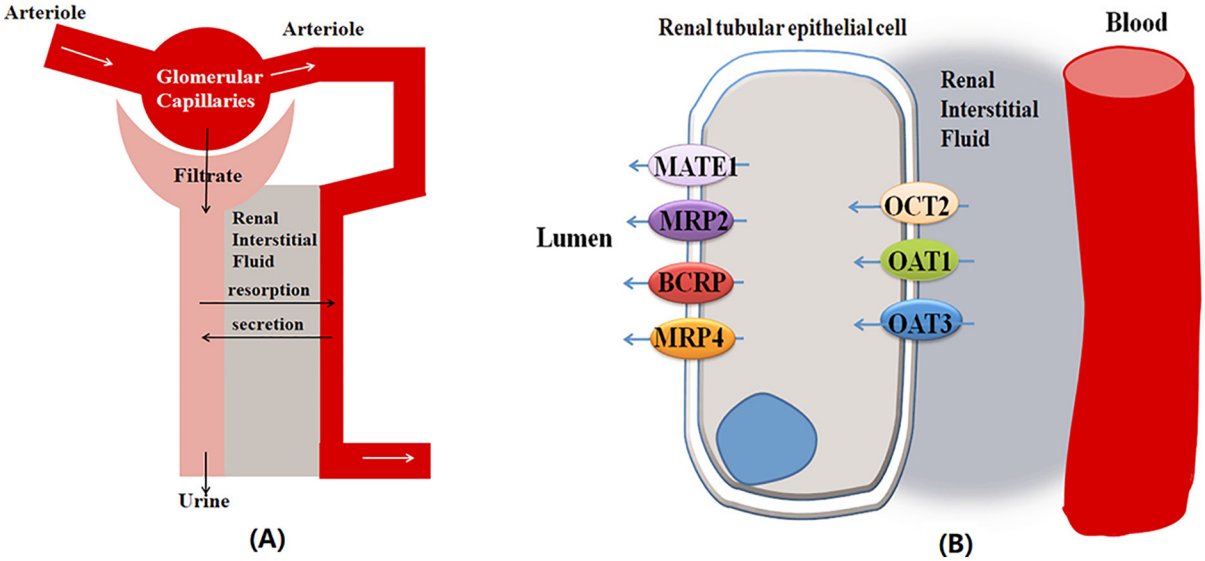

Fig. 1. Diagram of kidney. A: Process of urine excretion. Solutes are primarily excreted by glomerular filtration, tubular reabsorption and tubular secretion. B: Drug transporters in the kidney. Secretion transporters are responsible for the vector transport of substrates from the basolateral membrane to the apical membrane of proximal tubule cells. (OCTs: organic cation transporters; OATs: organic anion transporters; MRPs: multidrug resistance-associated proteins; BCRP: breast cancer resistance proteins; MATE1: multidrug and toxin extrusion 1).

fluid, electrolyte exchange, insulation and endocrine function [3,6]. Renal interstitial expansion is now considered as a useful marker for the progression of several nephropathies [7]. Additionally, renal fibrosis, particularly tubulointerstitial fibrosis, is a common final outcome of almost all progressive chronic kidney diseases [8]. Silva et al. have mentioned that it's difficult to establish whether tubular epithelial damage is secondary to interstitial nephritis or is the primary event [9]. Thus, renal interstitial injuries induced by drugs cannot be ignored.

From the histology of kidney, it can be seen that kidney is constructed by the components of the glomerulus, tubules, interstitium and blood vessels, and all exchanges among the tubules and vessels have to pass through the renal interstitium (shown in Fig. 1). Nishiyama et al. reported that the concentration of angiotensin II in renal interstitial fluid is much higher than that in the plasma, and that acute volume loading significantly reduced plasma solute concentration, but did not significantly reduce tubular fluid concentrations of solute [10]. Similar results were obtained by Eickenberg et al., who reported that the concentrations of antibiotic in the interstitial fluid were less than urinary levels but exceeded serum concentrations. In addition, three structurally unrelated antibiotics (cephalothin, ampicillin, tetracycline) that have different serum binding proteins had unchanged rates of clearance from renal interstitial fluid [11]. These evidences showed that the renal interstitium could be a compartmentalization independent of the circulating blood. Thus, we deduced that drugs could be trapped outside of the cell and could accumulate in the renal interstitium when cell uptake was inhibited, which could also be related to nephrotoxicity.

ADV is a prescription medicine used to treat hepatitis B viral infections, and is a drug that is predominantly cleared by renal tubular secretion. Renal interstitium fibrosis induced by ADV is reported in clinics [12]. In this work, the pharmacokinetics and nephrotoxicity of ADV in the renal interstitium were investigated.

2. Materials and methods

2.1. Animals

Eight-weeks-old Wistar rats weighing approximately 200–250 g were obtained from the Experimental Animal Center of Lanzhou University (Lanzhou, China, lzu20170320). Rats were housed in plastic cages in animal rooms with temperature-controlled (25 ± 2 °C) and were kept on a 12–12 h alternating light-dark cycle with free access to food and water. All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals and reagents

Adefovir (purity 98%) and probenecid (purity 98%) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Imatinib (internal standard of ADV) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol was high-performance liquid chromatography (HPLC)-grade (Fisher Scientific, NJ, USA). All other reagents used were analytical grade.

2.3. Study design

Study 1 investigated the urine excretion and kidney distribution of ADV. Rats were randomly divided into an ADV group and an ADV plus probenecid group (n = 6). Both ADV and probenecid were dissolved in sodium hydroxide solution and adjusted the PH to 7 with sodium acetate. Rats in the ADV group were orally administered ADV (10 mg/kg), while rats in the coadministration group were treated with ADV (10 mg/kg) and probenecid (30 mg/kg). Urine samples were collected at intervals of 0–2, 2–4, 4–6, 6–8, 8–10 and 10–12 h post-ADV administration. After a washout period of 1 week, rats were again treated as above, and then sacrificed 2 h post-ADV administration. Blood was collected via the abdominal aorta. The residual blood in the body was removed by saline cardiac perfusion. The left kidney was moved and separated into medulla and cortex by a careful dissection. Cortical wedges extended from the surface of the kidney to just above the red junctional zone between cortex and medulla. Medullary segments extended form just below the red junctional zone to the tip of the papilla. The right kidney was collected integrally. Then they were weighed and stored at −80 °C for ADV level determination.

Study 2 investigated the pharmacokinetics of ADV in the renal interstitial fluid. First, rats were randomly divided into two groups (n = 6), which were treated with either ADV (10 mg/kg) or ADV (10 mg/kg) plus probenecid (30 mg/kg) for 1, 7, 14, or 28 days. Renal interstitial fluid was collected by microdialysis (shown in 2.4 of materials and methods) in situ. Second, new groups of rats were divided and

treated as above, and then sacrificed 2 h post-ADV administration. Blood was collected via the abdominal aorta. The residual blood in the body was removed by saline cardiac perfusion. The left kidney was moved and separated into medulla and cortex. The right kidney was collected integrally. Then they were weighed and stored at −80 °C for ADV level determination. Third, new normal rats were randomly divided into three groups ($n = 6$). Kidney was isolated and perfused with a Krebs-Henseleit buffer containing ADV (47.25 μg/ml), high dosage ADV (141.75 μg/ml), or ADV (47.25 μg/ml) plus probenecid (100 μg/ml). Renal interstitial fluid was collected by microdialysis (shown in 2.4 of materials and methods) in an isolated kidney.

Study 3 aimed to test the effect of ADV on the expression of OATs/Oats. First, rats were treated with ADV for 1, 7, 14 or 28 days. 12 h after the final administration, the kidneys were removed and stored at −80 °C for Oats level determination. Second, HEK-293 cells were treated with 20, 40, 60 and 80 μg/ml doses of ADV for 7 h (at this time point and concentration, drug-induced morphological changes were observed without a large loss in cell numbers due to cell death). After treatment, the cells were lysed with a RIPA buffer supplemented with PMSF (phenylmethanesulfonylfluoride) (RIPA:PMSF 99:1, v/v). Proteins were extracted, denatured, and then stored at −80 °C for Oats level determination. The expression of Oats was investigated by western blotting.

Study 4 evaluated nephrotoxicity induced by ADV after long-term treatment. Rats were randomly divided into a control group, a probenecid group, an ADV group and an ADV plus probenecid group ($n = 8$). Rats in the control group were orally administration saline with sodium hydroxide solution and adjusted the PH to 7 with sodium acetate. Rats in the probenecid group were orally administered probenecid (30 mg/kg). Rats in the ADV group were orally administered ADV (10 mg/kg), and rats in the coadministration group were treated with ADV (10 mg/kg) and probenecid (30 mg/kg). The treatment was once a day for 1, 7, 14 or 28 days. 12 h after the final administration, bloods was collected via the abdominal aorta to determine biochemical parameters, and the residual blood was removed by a saline cardiac perfusion. Then, the kidneys were removed, and fixed with a 10% neutral buffered formaldehyde.

2.4. Renal microdialysis

Renal interstitial fluids were collected in situ using renal microdialysis. The left kidney was exposed via a flank incision. Then, a microdialysis probe (molecular weight cutoff point: 30000 Da, length: 4 mm) was inserted into the renal cortex of anesthetized rats and connected to a microinfusion pump that perfused the tissue with isotonic saline and heparin (30 units/ml) at a rate of 3 μl/min. Renal interstitial fluids from the microdialysis probes were collected at intervals of 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–7, 7–8, 8–9 and 9–10 h after administration of ADV or ADV plus probenecid.

Additionally, renal interstitial fluids were collected by microdialysis in an isolated kidney. As previously reported, after the rats were anesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg), polyethylene tubing were inserted into the right renal artery, vein and ureter, respectively, and then the kidney was removed [13,14]. Then, the microdialysis probe (molecular weight cutoff point: 30000 Da, length: 4 mm) was inserted into the renal cortex of the isolated kidney, which also was connected to a microinfusion pump to perfuse with isotonic saline and heparin (30 units/ml) at a rate of 1 μl/min. The isolated kidney was pre-perfused with a Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 27 mM NaHCO3, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 0.05 mM EDTA, and 5 mM D-glucose at a pH of 7.3–7.6) enriched with 95% O2 and 5% CO2 for 5 min at a flow rate of 40 ml/min/kg, and then perfused with a Krebs-Henseleit buffer containing ADV, high dosage ADV, or ADV plus probenecid at 37 °C. Renal interstitial fluids from the microdialysis probes were collected at intervals of 0–15, 15–30, 30–45 min after buffer administration.

The concentrations of ADV in the interstitial fluid were calculated according to the following equation:

$$V_{dead\ volume} = S_{\text{Internal surface area}} \times L_{outlet\ tube} \quad (1)$$

$$C_{dialysate\ 2-real} = \frac{C_{dialysate\ 2} \times V - C_{dialysate1} \times V_{dead\ volume}}{V - V_{dead\ volume}} \quad (2)$$

$$\text{RL} = \frac{C_{perfusate} - C_{dialysate}}{C_{perfusate}} * 100\% \quad (3)$$

$$C_{renal\ interstitial\ fluid} = C_{dialysate-real}/RL \quad (4)$$

where $V_{\text{dead volume}}$, $S_{\text{Internal surface area}}$ and $L_{\text{out tube}}$ are the dead volume, internal surface area and length of the outlet tube, respectively; $C_{\text{dialysate n}}$ is the ADV concentration in the dialysate determined by HPLC-MS/MS; V is the volume of the collected dialysate; $C_{\text{dialysate-real}}$ is the ADV concentration in the dialysate after mitigating dead volume; RL is the relative loss; $C_{\text{perfusate}}$ is the ADV concentration in the perfusate; and $C_{\text{renal interstitial fluid}}$ is the ADV concentration in the renal interstitial fluid.

2.5. Sample preparation and analysis

Blood samples were immediately transferred to a heparinized microcentrifuge tube and centrifuged at 18,000*g* for 10 min. Urine samples were diluted 10-fold with blank urine which were collected from rats in study 1 before experiment. Kidneys (0.5 g) were weighed and homogenized with 200 μl saline; then tissue samples were obtained after centrifugation at 18, 000*g* for 10 min. Interstitial fluid samples collected from the isolated perfused kidney did not need to be diluted. All of the above samples (50 μl) were vortex-mixed with 0.1 ml of acetonitrile containing imatinib (0.1 μg/ml) as the internal standard (IS) for 30 s. After 10 min the mixture was centrifuged for 5 min to separate precipitated proteins. A 2 μl aliquot solution was directly injected into the chromatography. The concentration of ADV was determined by an Agilent 1260 Infinity HPLC coupled to an Agilent 6460 Tripe-Quadrupole mass spectrometer equipped with the electro-spray ionization (ESI) interface. Multiple reactions monitoring mode was utilized to detect the compound of interest.

2.6. Biochemical analysis and histological evaluation

Blood samples were collected into a 10 ml capacity sample bottle and centrifuged at 716 *g* for 15 min. Serum creatinine, urea nitrogen, cystatin C, uric acid and P were determined by a fully automated chemistry analyzer (OLYMPUS AU2700; Olympus Co., Tokyo, Japan) according to the standards of the National Center for Clinical Laboratory (Beijing, China).

The kidney tissues in the control, and ADV groups were removed after the final administration, and fixed in a 10% neutral buffer formaldehyde solution. Segments were embedded in paraffin and stained with hematoxylin-eosin, sirius red or masson's trichrome by the standard procedure. A complete section of the kidney was screened at a magnification of 200 X, and the findings for the cortex were semi-quantitatively scored by three independent observers. The scoring systems used were adapted from a previous report on the pathological aspects of CHN and were defined as follows [15,16]. Tubular atrophy: 0, normal tubules; 1, a rare single atrophic tubule; 2, several clusters of atrophic tubules; 3, massive atrophy. Tubular necrosis: 0, normal tubules; 1, rare single necrotic tubule; 2, several clusters of necrotic tubules; and 3, massive necrosis. Interstitial fibrosis: 0, absent; 1, minimal fibrosis, with slight thickening of the tubular basal membrane; 2, moderate fibrosis, with focal enlargement of the interstitium; and 3, severe fibrosis, with confluent fibrotic areas. Tubulointerstitial injury scores were defined as the sum of the three aforementioned scores.

2.7. Western blot assay

The kidney tissues and HEK 293 cells were homogenized in the RIPA buffer, and then centrifuged at 4 °C. Supernatants were collected, and the protein content of the supernatants was measured by BCA. The protein samples were denatured at 95 °C for 5 min. Western blot was performed using Abcam standardized techniques. Anti-OAT1(ab131087) and anti-beta actin were purchased from Abcam Inc. Anti-OAT3(sc98807) was purchased from Santa Cruz Biotechnology. The scanned digital images were quantified using NIH ImageJ software.

2.8. Statistical analysis

The data are expressed as the mean ± SD, and statistically significant differences of data from two sets were compared using a one-way analysis of variance. In all statistical analyses, $p < 0.05$ or $p < 0.01$ was considered statistically significant.

3. Results

3.1. LC-MS/MS conditions

The selected transitions were *m/z* 274.3 to *m/z* 88.0 for ADV and *m/z* 494.3 to *m/z* 394.3 for IS. In addition, the concentrations of urotoxins were determined by LC-MS/MS. The selected transitions were *m/z* 206.8 to *m/z* 81.7 for Uridine, *m/z* 209.1 to *m/z* 145.9 for NG-NG'Dimethy-L-arginine, *m/z* 236.6 to m/z 145.4 for N2-Acetyl-L-arginin, *m/z* 114 to *m/z* 44.2 for creatinine, *m/z* 318 to *m/z* 256.1 for 3-Deoxyglucosone, *m/z* 212.4 to *m/z* 80 for Indole sulfate potassium sulfate, *m/z* 174.4 to *m/z* 120 for Indole-3-acetic acid and *m/z* 178.1 to *m/z* 134 for 2H-Pyrrolo[3,4-*d*]isoxazole-6-carboxylicacid and *m/z* 494.3 to *m/z* 394.3 for IS. The described method has been successfully used to determine the concentration of ADV. Under these optimum conditions, the calibration curves had good linearity within the linear range, and the values of accuracy and precision were within the recommended limits.

3.2. The effect of probenecid on the urinary excretion of ADV

As shown in Fig. 2A, the 12 h cumulative urinary excretion of ADV was decreased by 21.89% in ADV plus probenecid group compared to that in ADV group. However, the ADV concentration in the kidney, shown in Fig. 2B, was unexpectedly increased by probenecid. The renal tissue-to-plasma ratio of ADV concentration in the ADV plus probenecid group was lower than that in the ADV group. The plasma concentration of ADV in the ADV plus probenecid group was significantly elevated compared to that of the ADV group 2 h after oral administration of ADV.

3.3. The pharmacokinetics of ADV in the renal interstitium

The mean interstitial concentration-time curves of ADV after oral treatment with ADV or ADV plus probenecid are shown in Fig. 3. The ADV concentrations in the renal interstitial fluid, after treatment with ADV plus probenecid for 1, 7, 14 or 28 days were all increased compared to those of the ADV group. The relevant pharmacokinetic parameters are listed in Table 1. Compared to those in the ADV group, the AUC and C_{max} in all ADV and probenecid coadministration groups increased, whereas those of the CL were all decreased. Significant differences of AUC and C_{max} were observed after treatment for 7, 14 and 28 days. Significant differences of CL were observed after treatment for 7 and 14 days. In addition, the CL of ADV in the ADV groups was decreased dependent on the time, while the AUC of those were increased dependent on the time. Similar results were observed in the ADV plus probenecid groups. Furthermore, the concentration of ADV in plasma and kidney were all decreased in the ADV plus probenecid group compared to the ADV group (Shown in Fig. 4A) and the renal tissue-to-plasma ratio of ADV concentration in the ADV plus probenecid group was higher than that in the ADV group after treatment for 28 days (Shown in Fig. 4B), which indicated that ADV could have accumulated in the kidney when Oats were inhibited.

3.4. The effect of ADV on the protein expression of OAT1 and OAT3

The expression of OAT1/Oat1 and OAT3/Oat3 in rat kidneys and HEK293 cells are shown in Fig. 5. In rats, the expression of Oat1 was significantly decreased after treatment with ADV for 28 days, while the expression of Oat3 was significantly decreased after treatment with ADV for 14 and 28 days. The expression of OAT1 and OAT3 in HEK 293 cell were decreased at the concentrations of 40 and 80 μg/ml. These results may explain why ADV concentration in renal interstitium could also increase with the treatment time.

3.5. The effect of probenecid on the ADV concentration in renal interstitium

To confirm the effect of Oats on the ADV concentration in renal interstitium, renal interstitial fluids were collected from an isolated kidney that was perfused with a Krebs-Henseleit buffer containing ADV, high dosage ADV, or ADV plus probenecid. As shown in Fig. 6, the ADV concentrations in the renal interstitial fluid in ADV plus probenecid group or high dosage ADV group were both significantly increased compared to that of the ADV group. This result indicated that ADV concentration in the renal interstitial fluid could be increased by either Oats inhibition or high concentration of ADV in plasma.

3.6. Biological parameters and uremic toxins for long-term treatment with ADV or ADV plus probenecid in rats

Serum biochemistry results are shown in Fig. 7A. No statistically significant differences were found between any of the groups in terms of serum creatinine, uric acid, nitrogen, phosphate or cystatin C after 7 days of treatment. After 14 days of treatment, serum phosphate and cystatin C levels were significantly higher in the ADV plus probenecid group than in control group ($P < 0.05$), but this difference was not present after treatment for 28 days. After 14 days of treatment, uric acid and nitrogen levels in the ADV plus probenecid group increased significantly compared with the control group ($P < 0.05$). After 28 days of treatment, uric acid levels in the ADV and ADV plus probenecid groups both increased significantly compared to that of the control group, and nitrogen levels in the ADV plus probenecid group increased significantly compared with those of the control group ($P < 0.01$) or the ADV group ($P < 0.01$).

As shown in the Fig. 7B, the kidney concentration of 3-Deoxyglucosone and orotic acid in the ADV plus probenecid group were increased significantly compared to the ADV group. The plasma concentrations of the N2-Acetyl-L-arginin, uridine, NG-NG'Dimethy-L-arginine and 2H-Pyrrolo[3,4-*d*]isoxazole-6-carboxylicacid in the ADV plus probenecid group were higher than that in the ADV group. They were not detected that the concentrations of uremic toxins in renal interstitial fluid.

3.7. Histological findings and scores

The sections were stained with sirius red and photographed in polarized light. As show in Fig. 8A, collagen fibers polarization colors were found to be yellowish-orange in control and ADV group. In the ADV plus probenecid group, collagen fibers polarization colors were greenish-yellow predominantly. Masson stain showed that interstitial fibrosis was present obviously in the ADV plus probenecid group (28 days), whereas no trace of interstitial fibrosis could be detected in the ADV groups (Fig. 8B).

Scores for tubular atrophy, tubular necrosis, and interstitial fibrosis

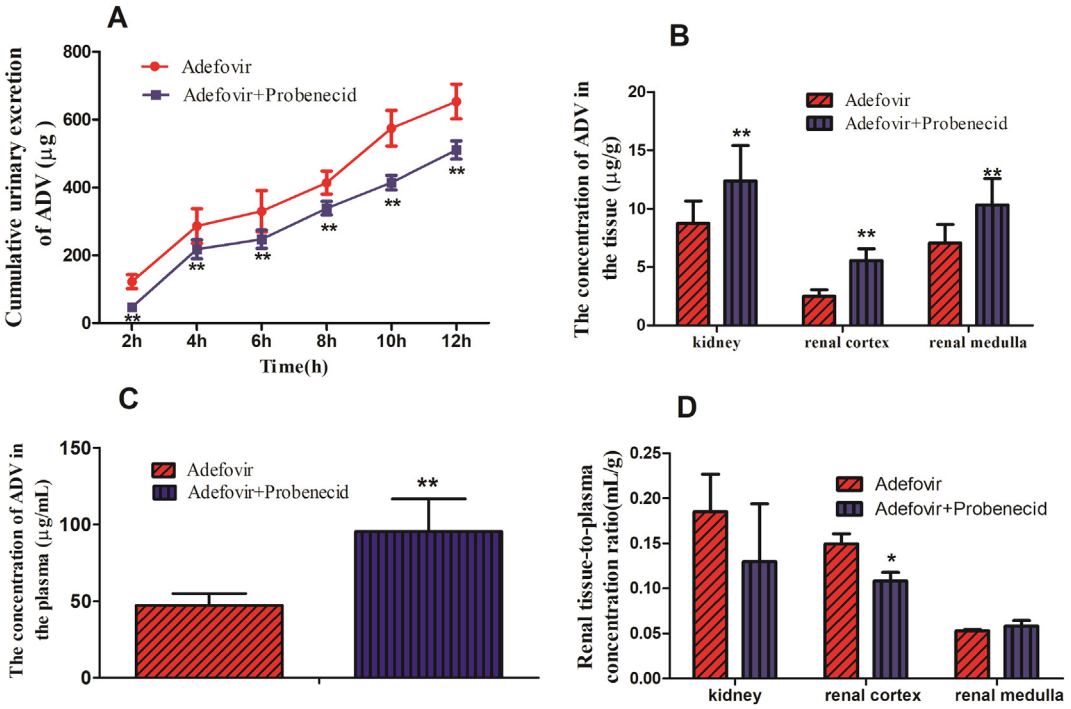

Fig. 2. The effect of probenecid on urine excretion, plasma concentration and kidney distribution of ADV. (A)12 h cumulative urinary excretion of ADV, (B) concentration of ADV in the kidney. (C) concentration of ADV in the plasma. (D) renal tissue-to-plasma ratio of ADV concentration. Data are expressed as Mean ± S.D., $n = 6$. (Compared with the ADV group, * $p < 0.05$, ** $p < 0.01$).

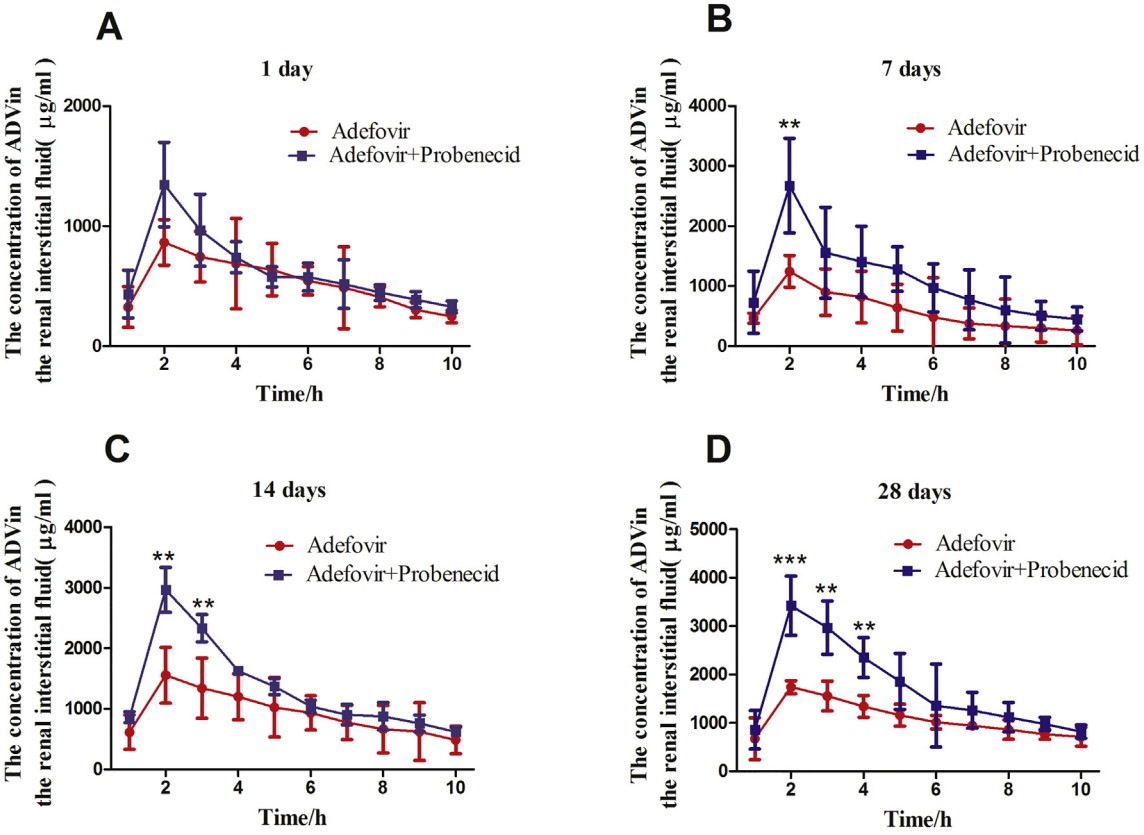

Fig. 3. The effect of probenecid on the pharmacokinetic profile of ADV in the renal interstitial fluid after treatment for 1 day, 7,14, or 28 days in rats. Data are expressed as mean ± S.D., n = 6.

Table 1
The effect of probenecid on the pharmacokinetic parameters of ADV in the renal interstitial fluid after treatment for 1 day, 7, 14, or 28 days in rats. Data are expressed as the mean ± S.D., n = 6.

		AUC (μg/mL*min)	CL (mL/min/g)	$t_{1/2}$(h)	C_{max} (μg/mL)
1 Day	ADV	5127.5 ± 878.3	1.92 ± 0.205	2.982 ± 0.545	863.78 ± 134.25
	ADV + Probenecid	6154.5 ± 1136.1	1.435 ± 0.547	5.015 ± 2.89	1345.85 ± 119.04 [b]
7 Day	ADV	5696.86 ± 606.7	1.504 ± 0.17	3.91 ± 1.64	1244.45 ± 46.28
	ADV + Probenecid	10,734.4 ± 561.9 [b]	0.889 ± 0.068 [b]	5.66 ± 0.17	2675.46 ± 92.24 [b]
14 Day	ADV	8989.4 ± 408.8	0.923 ± 0.068	4.89 ± 2.04	1557.64 ± 66.78
	ADV + Probenecid	13,402.3 ± 80.97 [b]	0.58 ± 0.17 [a]	7.87 ± 6.11	2966.96 ± 101.44 [b]
28 Day	ADV	10,406.7 ± 199.7	0.65 ± 0.14	5.26 ± 1.67	1738.23 ± 84.76
	ADV + Probenecid	16,570.2 ± 246.7 [b]	0.513 ± 0.068	8.17 ± 3.12	3419.78 ± 76.49 [b]

Compared with the ADV group, a < 0.05, b < 0.01.

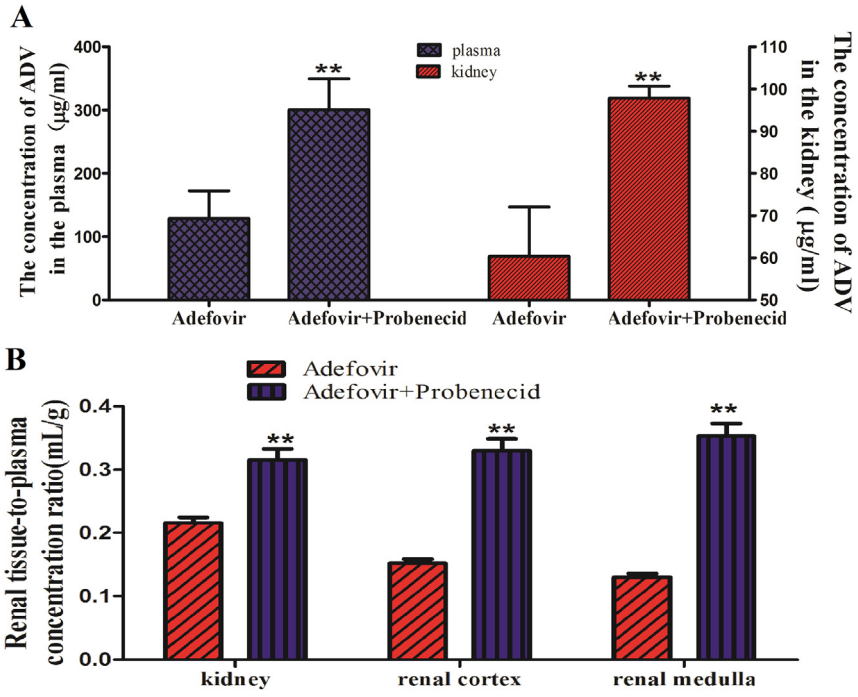

Fig. 4. The effect of probenecid on plasma concentration and kidney distribution of ADV after treatment for 28 days. (A) concentration of ADV in the plasma or kidney. (B) ratio of renal tissue-to-plasma ratio of ADV concentration. Data are expressed as Mean ± S.D., n = 6. (Compared with the ADV group, * p < 0.05, ** p < 0.01).

were calculated from individual data collected for each group. The histological structure was shown in Fig. 8C. Rats in the control and probenecid groups didn't exhibit renal morphological alterations. In the ADV group, no remarkable lesions were observed, except for slight tubular atrophy and a few instances of tubular necrosis. In contrast, along with tubular atrophy and tubular necrosis, moderate fibrosis with focal enlargement of the interstitium was also observed in the ADV plus probenecid group after treatment for 28 days. The scores in the ADV plus probenecid group were significantly higher than those in the ADV group (Fig. 8D).

4. Discussion

In the past decade, research has indicated that nephrotoxicity induced by drugs may be caused either by an excessive cell uptake or by a decreased extrusion outside of the proximal tubular cells [17], and reduced cell uptake could protect against nephrotoxicity and decrease renal clearance [1,18,19]. However, some studies have found that reduced cell uptake couldn't protect against nephrotoxicity. For example, it has been confirmed that cisplatin induced nephrotoxicity related to cell uptake mediated by organic cation transporters (OCTs). However, although the severity of cisplatin induced nephrotoxicity was reduced following genetic or pharmacologic knockout of Oct1 and Oct2, renal tubular damage wasn't completely abolished [19]. Furthermore, one study indicated that OCTs inhibitors, such as cimetidine, enhanced cisplatin toxicity in mice [20]. It has also been observed that the nephrotoxicity of tenofovir and methotrexate (tubular epithelium uptake mediated by OATs) was increased by nonsteroidal anti-inflammatory drugs, which are also OATs inhibitors [21,22]. Researchers have considered that inhibitors of uptake transporters located in the basolateral membrane of the tubular epithelium could also inhibit efflux transporters located in the luminal membranes of the tubular epithelium, and the inhibitory effect of uptake transporter inhibitors on efflux transporters could be stronger than that on the uptake transporter [23–25]. Thus, the reduction of cell uptake couldn't protect against nephrotoxicity might be associated with decreased expulsion mediated by efflux transporters alternatively. However, the intracellular concentration of drug has never been studied in vivo. It is unknown that whether mutations in genes or the inhibition of efflux transporters could also cause the accumulation of potential nephrotoxins in tubular cells. Servais A et al. found that the mutation of multidrug resistance-associated proteins (MRP) 2 alone in efflux transporters did not affect ADV clearance, which suggested that MRP2 did not play a critical role in the secretion of ADV [26]. Rokx C. et al. reported that concomitant incidental exposure of MRPs inhibitors and tenofovir did not result in major additional tenofovir-related renal toxicity in HIV-infected patients [27]. These results indicated that other mechanisms that contribute to drug-induced renal damage existed, which were independent of the transporters mediated accumulation of drugs in tubular cells.

According to the histology of the kidney, we hypothesized that

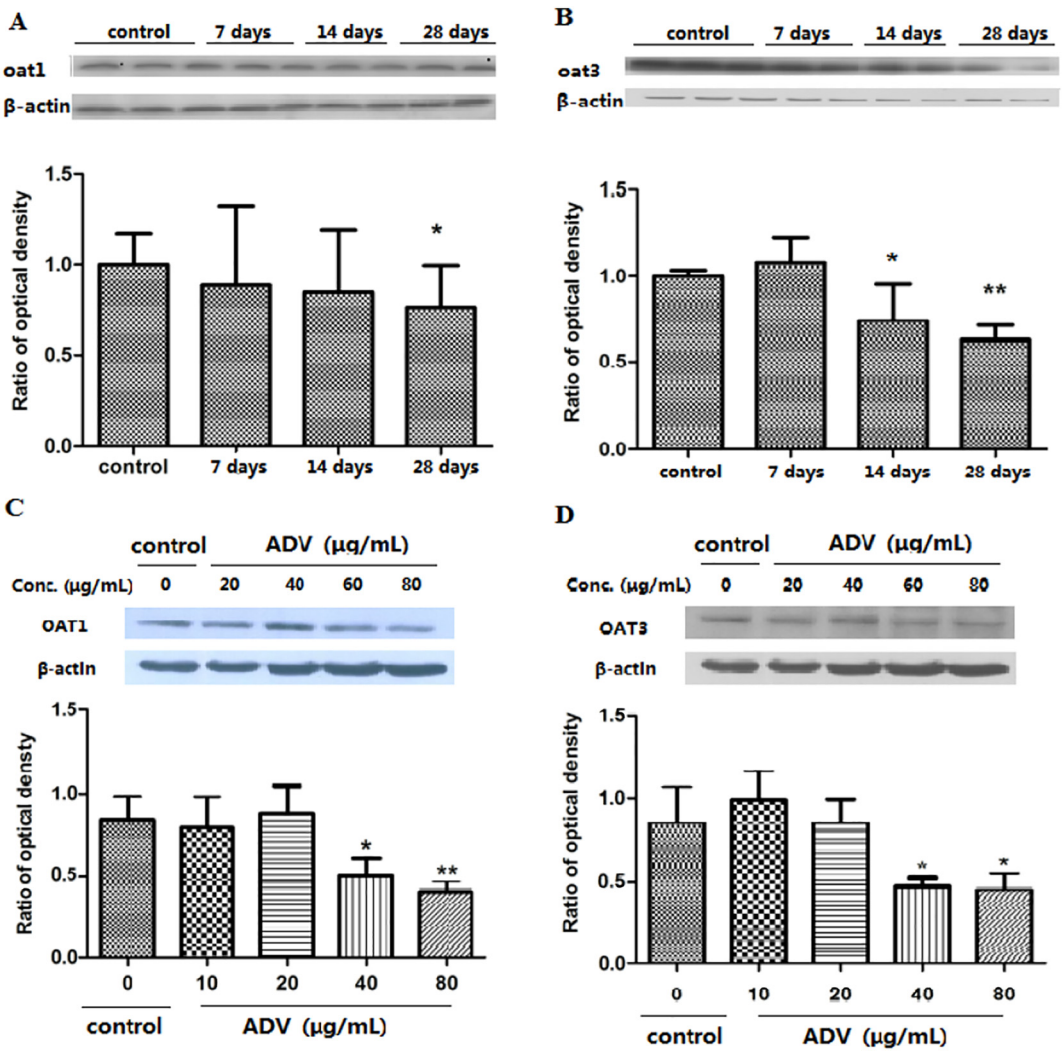

Fig. 5. The expression of Oat1/OAT1 and Oat3/OAT3 in rat kidneys (A, B) or HEK293 cells (C, D). Data are expressed as the mean ± S.D., $n = 3$. (Compared with the control group, * $p < 0.05$, ** $P < 0.01$).

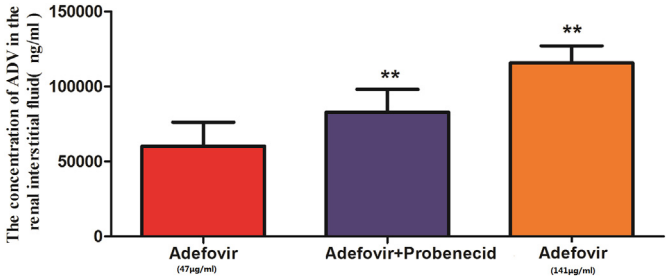

Fig. 6. The concentration of ADV in the renal interstitial fluid after treatment with ADV (47.25 μg/ml), ADV+ probenecid (47.25 μg/ml and 100 μg/ml, respectively) or high dosage ADV (141.75 μg/ml) in an isolated kidney. Data are expressed as the mean ± S.D., n = 6. (Compared with the ADV group, * $p < 0.05$, ** $P < 0.01$).

drugs could be trapped outside of the cell and accumulate in the renal interstitium when cell uptake was inhibited, which could be related to nephrotoxicity. In this work, we found that the cumulative urinary excretion of ADV could be reduced by the Oats inhibitor probenecid, while the ADV concentration in the kidney was unexpectedly increased by probenecid. The renal tissue-to-plasma ratio of ADV concentration couldn't be reduced by probenecid. The results showed that the concentrations of ADV in the renal interstitial fluid from the in vivo kidneys were increased in the ADV plus probenecid group compared to the ADV group. To avoid the effects of an increased ADV concentration in the plasma on the concentration of ADV in the interstitial fluid, the concentration of ADV in the renal interstitial fluid was collected by microdialysis in an isolated kidney. The results showed that the ADV concentration in the renal interstitial fluid could also be increased by the Oats inhibitor probenecid. Consistent with previous studies, our results found that the concentration of ADV in the interstitial fluid was higher than that in the plasma. Thus, ADV could accumulate in the renal interstitium when Oats were inhibited. Lemley et al. have indicated that the renal interstitium is the intertubular and intercapillary space that is occupied by fibroblasts, dendritic cells, a network of collagen fibers, hyaluronic acid, glycoproteins, and interstitial fluid and structured as a gel that supports tubules and capillaries by virtue of its resilience [28]. Therefore, the movement of solutes in the renal interstitium could be slow and limited, which may explain why ADV could accumulate there.

After treatment with ADV or ADV plus probenecid for 1, 7, 14 or 28 days, the AUC_{0-10} of ADV in the interstitial fluid were increased dependent on time, which could be attributed to the decreased expression of OAT1/Oat1 and OAT3/Oat3 in HEK293 cells or in rats after treatment with ADV. However, our results showed that interstitial fibrosis was present in the ADV plus probenecid group after treatment for 14 and 28 days, whereas no trace could be detected in the ADV groups.

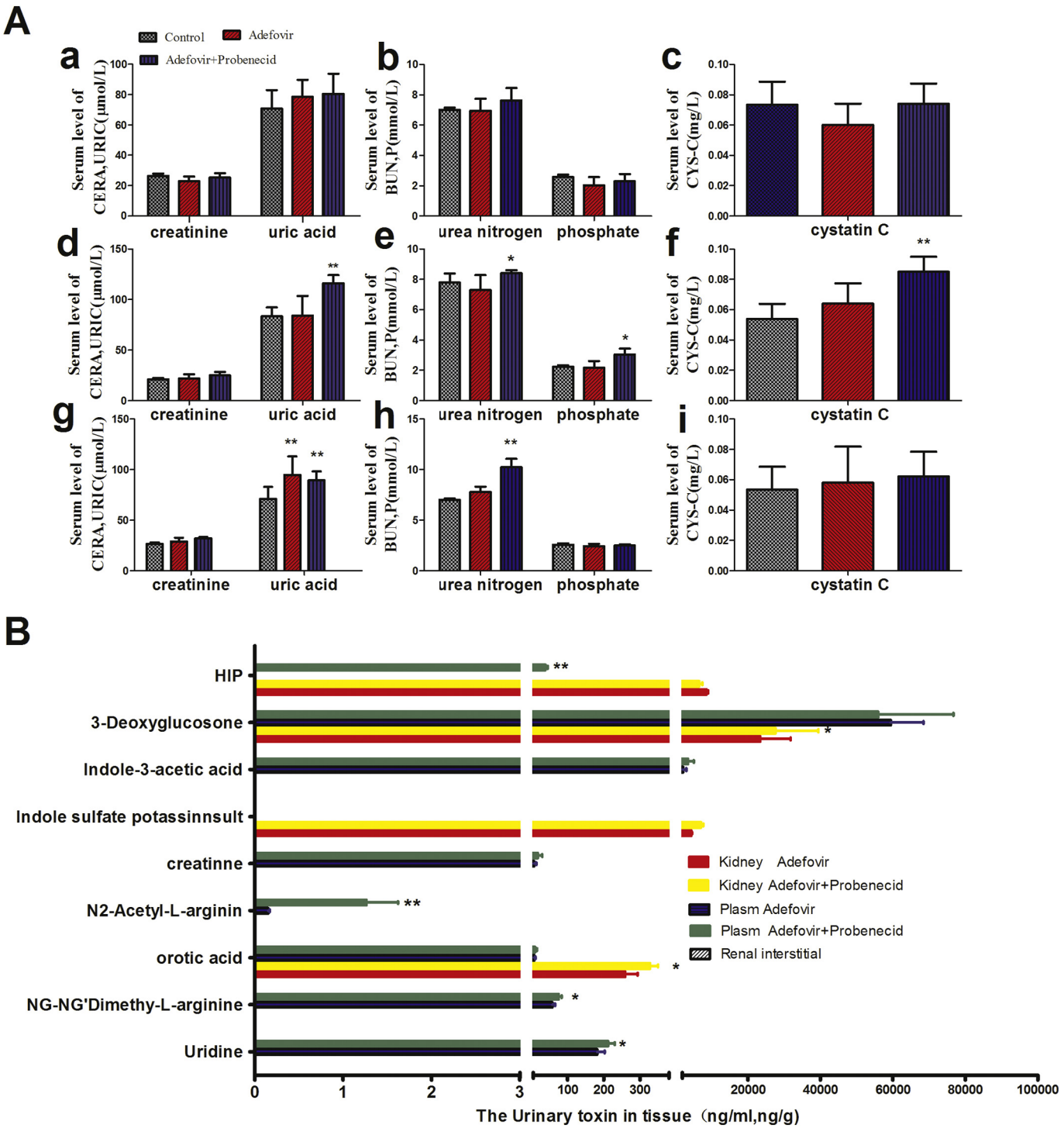

Fig. 7. A: Biological parameters in serum after treatment with ADV or ADV plus probenecid for 7 (a, b, c), 14 (d, e, f) or 28 (g, h, i) days in rats. B: uremic toxins in kidney, plasma or interstitial fluid (Compared with the control groups, * $p < 0.05$, ** $P < 0.01$, $n = 8$.)

Tubular injury was observed in the ADV plus probenecid group, and was found to be even stronger than that in the ADV group. The interstitium is bounded on all sides by tubular and vascular basement membranes [29]. Thus, the concentration of ADV in the renal interstitium might play an important role in tubulointerstitial injuries, and nephrotoxicity could be caused by the accumulation of drugs in the renal interstitium when the function of uptake transporters is inhibited.

In fact, we think the idea previously accepted concepts that nephrotoxicity could be protected against by the inhibition of cell uptake is not conflict with our results. Drug-induced nephrotoxicity is increasingly recognized as a significant contributor to kidney disease including acute kidney injury (AKI) and chronic kidney disease (CKD). Generally, AKI is often caused by short-term drug treatment, which could be attributed to the accumulation of drugs in proximal tubule cells, AKI could be protected against by the inhibition of cell uptake and the reduction of drugs concentration in the tubular epithelium. Since the renal interstitium has a stronger compensatory ability, CKD is usually caused by long-term drug treatment of drugs. The inhibition of cell uptake would lead to drug accumulation in the renal interstitium for long periods of time and cause nephrotoxicity.

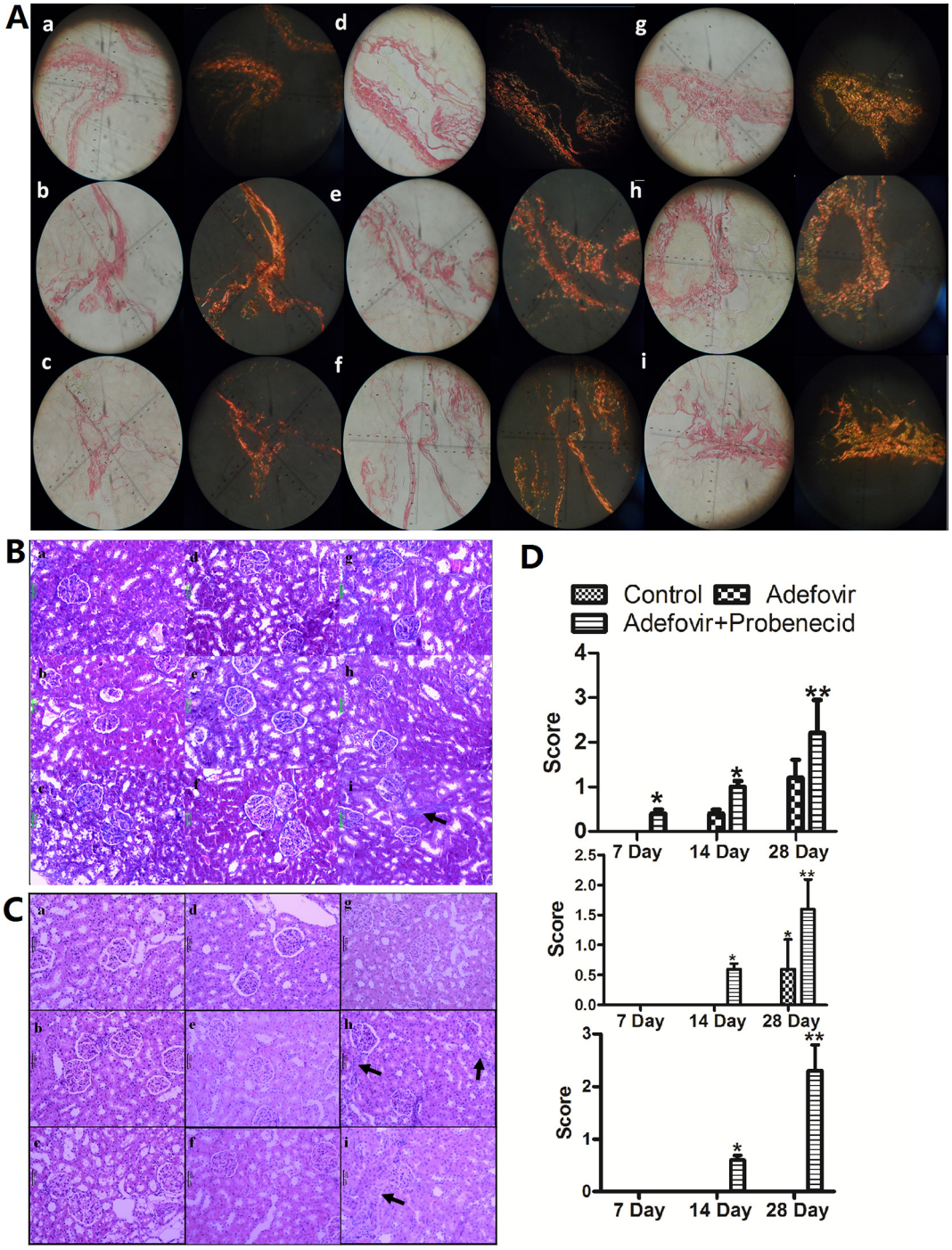

Fig. 8. Histological structure analysis. A: sirius red stain and photographed in polarized light. B: Masson stain; C: HE stain; D: semiquantitative tubulointerstitial scores of kidneys. a-c, d-f or g-i were treated with saline, ADV or ADV plus probenecid for 7,14 and 28 days, respectively. Interstitial fibrosis are indicated in B and C by arrows. (Compared with the control groups, * $p < 0.05$, ** $P < 0.01$, $n = 5$.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In conclusion, the renal interstitium is a central determinant of the fate of kidneys disease because it plays an integral role in physiological kidney function [3]. Our study indicated that ADV could be accumulated in the renal interstitium after Oats inhibition and related with nephrotoxicity. Thus, to find an effective and safe strategy to protect against nephrotoxicity induced by drugs, we should pay more attention to the interstitial injury induced by drugs.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant no. 81603211) and the Foundation of the First Hospital Lanzhou University (Grant no. ldyyyn2018-06).

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