Mechanism of dioscin ameliorating renal fibrosis through NF-κB signaling pathway-mediated inflammatory response

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Abstract. Dioscin (DIS) is a natural compound derived from Chinese herbal medicine. In recent years, multiple studies have reported that DIS has immunoregulation, anti-fibrosis, anti-inflammation, anti-viral and anti-tumor effects. However, the mechanism by which DIS ameliorates renal fibrosis and inflammation remains to be elucidated. The aim of the present study was to investigate the role of DIS in renal fibrosis and inflammation and to explore its underlying mechanism. It used network pharmacology to predict the targets of DIS for the treatment of renal interstitial fibrosis. The present study was performed using unilateral ureteral obstruction mice and HK-2 cells *in vivo* and *in vitro*. The mice were treated with different doses of DIS. Kidney tissues were collected for histopathology staining, western blotting, immunohistochemistry staining and reverse transcription-quantitative (RT-q) PCR. TGF-β1 (2 ng/ml) was used to induce renal fibrosis in the cells. Then, cells were respectively treated with DIS (3.125, 6.25, 12.5 μM) and Bay11-7082 (an inhibitor of NF-κB p65 nuclear transcription, 1 μM) for another 24 h. The expressions of inflammatory factors and NF-κB pathway proteins were detected by immunofluorescence, ELISA, western blotting and RT-qPCR. DIS alleviated renal injury in the UUO mice. Mechanistically, DIS not only decreased the expressions of inflammatory factors including IL-1β, NOD-like receptor thermal protein domain associated protein 3, monocyte chemotactic protein 1, IL-6, TNF-α and IL-18 but also reduced the level of phosphorylation of NF-κB p65 *in vivo* and *in vitro*, which was similar to the impact of Bay11-7082. DIS ameliorated renal fibrosis by inhibiting the NF-κB signaling pathway-mediated inflammatory response, which may be a therapeutic pathway for delaying chronic kidney disease.

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Introduction

Due to the prevalence of hypertension, diabetes, obesity and other diseases that cause kidney damage, the incidence of chronic kidney disease (CKD) is also on the rise year by year and has been one of the most serious illnesses that jeopardize human health (1). CKD is a progressive disease in which irreversible damage to the function and structure of the kidney occurs gradually over months or years. Renal fibrosis is an important pathological alteration in the progression of CKD to end-stage renal disease. Fibrosis is a repair of impairment; however, pathological fibrosis can cause organ dysfunction (2). The degree of renal fibrosis is also considered to be closely related to the prognosis (3). Thus, it was deemed extremely important to investigate effective treatment for renal fibrosis.

Renal fibrosis is mainly divided into two parts: glomerular fibrosis and interstitial fibrosis (4) and the present study only focused on renal interstitial fibrosis, which is characterized by infiltration of inflammatory cells and deposition of collagen fiber with various degrees of renal insufficiency. The mechanisms of renal interstitial fibrosis are generally thought to be related to inflammatory responses, activation and proliferation of fibroblasts, accumulation of extracellular matrix (ECM), epithelial-mesenchymal transition (EMT) (5), agonism of the renin-angiotensin system (RAS) and metabolic abnormalities, for instance, both an excess of parathyroid hormone and a deficiency of vitamin D can contribute to the progression of renal fibrosis in the patients with CKD (2,3,6). In recent years, the correlation between inflammatory response and renal fibrosis has received increasing attention. The inflammatory response is a critical part of renal interstitial fibrosis, which

is mainly accomplished by two types of inflammatory cells, macrophages and lymphocytes (4,7,8). In addition to secreting inflammatory mediators that exacerbate tissue injury, they also encourage fibroblast activation and accumulation of ECM (3). In unilateral ureteral obstruction (UUO) mice, knocking out the recombinant activator 1 protein hinders lymphocyte maturation attenuated renal fibrosis injury. Conversely, renal fibrosis is appreciably worse after lymphocyte importation (9). During the progression of acute kidney injury to CKD, inhibition of macrophage secretion significantly attenuated renal fibrosis (10). Li *et al* (5) demonstrate that renal fibrosis injury is alleviated following the suppression of the inflammatory response. These previous studies confirmed that inhibition of the inflammatory response reduced renal interstitial fibrosis.

Dioscin (DIS) is an active ingredient of Dioscoreaceae herbs with a number of biological activities (11). Its pharmacological effects are extensive, mainly involving the heart, liver, lung, kidney and other organs, with anti-inflammatory, anti-fibrotic, anti-tumor, anti-atherosclerotic, immunomodulatory and modulating oxidative stress responses (11-15). The regulation of the NF-κB pathway can trigger inflammatory responses (16). Studies have found that DIS can diminish the expression of inflammatory factors by inhibiting the phosphorylation of NF-κB p65 and thus suppress the inflammatory response (13,14). In addition, it has been found that DIS could reduce renal fibrosis by upregulating the Sirt3 signal (17). However, it is unclear whether DIS alleviates renal fibrosis by reducing NF-κB signaling pathway-mediated inflammatory response. The present study analyzed the targets of DIS action on renal fibrosis by network pharmacology (as shown in the Fig. S1), which included the NF-κB signaling pathway. The purpose of the current study is to determine how DIS affects renal fibrosis and inflammation, as well as potential underlying mechanisms.

Materials and methods

Identification of therapeutic targets for DIS in renal interstitial fibrosis. To investigate the effect of DIS on renal interstitial fibrosis, the present study used network pharmacology to predict the targets of DIS. PubChem (https://pubchem.ncbi.nlm.nih.gov/) was used to get the structured and SMILE files of DIS. The SMILE files were imported into SwissTargetPrediction (http://www.swisstargetprediction.ch/) and TargetNet (http://targetnet.scbdd.com/) to search the potential targets. The keywords 'renal interstitial fibrosis' were entered into DrugBank (https://go.drugbank.com), CTD (http://ctdbase.org), Genecards (http://www.genecards.org), DisGeNET (https://www.disgenet.org) and OMIM databases (http://omim.org) to collect the targets of disease. Then, the process of DIS on renal fibrosis was analyzed using Kyoto Encyclopedia of Genes and Genomes pathway analysis in Metascape database (http://www.metascape.org/). Significant pathways with $P<0.05$ were chosen.

UUO mice models and DIS treatment. To determine the sample size for each group of mice, the present study reviewed studies reporting on UUO mice and noted the high mortality rate of mice when UUO surgery was performed on the mice. The kidneys of the mice were intended for western blotting, reverse transcription-quantitative (RT-q) PCR and histopathological staining. Due to the amount of mouse kidney tissue required for these analyses, it decided to set the sample size at 10 mice per group, based on the research paper by Cao *et al* (18) and Gu *et al* (19).

Male C57BL/6 mice from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. weighing 17-22 g. The mice were kept in a cage with a 12-h light/dark cycle, a standard feed and unlimited access to water at a temperature of 22±2˚C and humidity of 40±5%. The bedding of the cages was changed daily and the condition of the mice was checked daily. The animal study was reviewed and approved by the Ethics Committee of the China-Japan Friendship Institute of Clinical Medical Sciences (approval no. zryhyy21-22-01-09; affiliated with the China-Japan Friendship Hospital, Beijing, China).

A total of 80 mice were randomly assigned to one of four groups (n=20/group), which included the sham-operated group, the UUO group, the UUO + 50 mg/kg DIS group and the UUO + 100 mg/kg DIS group. After three days of adaptive feeding, a model of interstitial renal fibrosis was prepared in mice by using UUO surgery. Pentobarbital (1%; 40 mg/kg) was used 1% to anesthetize mice by intraperitoneal injection before the surgery. The unilateral ureteral obstruction surgery was described previously (20). The sham-operated group underwent a sham operation. The mice were fasted for 12 h but were given free access to drinking water before the operation. The DIS group was administered from the first day after surgery and gavaged with DIS 50 mg/kg or 100 mg/kg every 24 h for 7 days. DIS was purchased from Herbpurify company (CAS: 19057-60-4, purity >98.0%, ID: S-048, Chengdu Herbpurify Co., Ltd.). The sham-operated and the UUO groups were orally administered physiological saline of equal volumes every 24 h for 7 days. Half of the mice were sacrificed on the 3rd and 7th day of treatment to obtain kidney tissue, respectively. The mice were fasted for 12 h before sacrifice. Pentobarbital sodium (1%; 50 mg/kg) by intraperitoneal injection was used, followed by intracardiac exsanguination. Animal mortality was verified by the absence of a heartbeat and respiration for >3 min.

Determination of urine protein and urine creatinine in mice. At the end of the experiment, urine in mice was collected. Urine protein was detected by mouse albumin ELISA kit (Bethyl Laboratories, Inc.). Urine creatinine was detected by an automatic biochemical analyzer. Then, the ratio of urine protein to urine creatinine was calculated.

Cell culture and pharmaceutical treatment. Human renal tubular epithelial cells (HK-2) were obtained from Professor HY Lan (Chinese University of Hong Kong) and placed in a cell incubator at 37˚C and 5% CO_2. The cells were cultured with DMEM/F-12 medium (Corning, Inc.), 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1X double antibiotics (penicillin and streptomycin). TGF-β1 (2 ng/ml) was used to induce renal fibrosis model in HK-2 cells. Then, the cells were respectively treated with DIS (3.125, 6.25, 12.5 μM) and Bay11-7082 (1 μM; cat. no. B5556; MilliporeSigma) for another 24 h. DIS (CAS: 19057-60-4; purity >98.0%; cat. no. SD8350) was purchased from Beijing Solarbio Science & Technology Co., Ltd.

Cell viability analysis. CCK-8 assay (Mei5 Biotechnology, Co., Ltd.) was used to evaluate DIS on HK-2 viability. HK-2 cells were seeded and grown in a 96-well plate for 24 h at 37˚C. Subsequently, the cells were treated with DIS for indicated periods (24, 48 and 72 h), respectively. Then the cells were incubated for 1 h with 10 μl of CCK-8 solution (5 mg/ml). An enzyme marker was used for analyzing cell absorbance at 450 nm.

Histopathological and immunohistochemistry staining. Kidney tissue specimens from mice were fixed with 10% formalin solution overnight at 4˚C and were dehydrated by ethanol. These specimens were then embedded in paraffin and were cut into 2-3-μm sections by a routine procedure (21). The sections were stained with hematoxylin and eosin (HE) and Masson's trichrome as described previously (22). Briefly, for HE staining, the sections were stained with hematoxylin for 5 min followed by eosin for 3 min, both at room temperature. For Masson's trichrome staining, the sections were stained with hematoxylin for 5 min, lichon red acidic magenta solution for 10 min, 1% phosphomolybdic acid solution for 5 min and aniline blue solution for 5 min. All Masson's trichrome staining steps were conducted at room temperature. All stained sections were viewed by a light microscope (Olympus BX53; Olympus Corporation).

The sections were routinely dewaxed and hydrated with 3% H_2O_2 solution for 10 min and rinsed three times with distilled water. Subsequently, the antigen was retrieved using a microwave oven. The sections were heated in a microwave oven on high with 0.01 M sodium citrate buffer for 10 min, cooled and rinsed once with PBS buffer. The blocking solution was added dropwise for 20 min. Primary antibodies against TNF-α (1:50; cat. no. sc-52746; Santa Cruz Biotechnology, Inc.), alpha-smooth muscle actin (α-SMA; 1:50; cat. no. 19245; Cell Signaling Technology, Inc.) and fibronectin (FN; 1:50; cat. no. ab2413; Abcam) were added dropwise, incubated for 2 h at room temperature and then rinsed 5 times for 2 min each using PBS buffer. The secondary bio-goat anti-mouse IgG and bio-goat anti-rabbit IgG antibodies (1:50 dilution; both from the immunohistochemistry staining kit; cat. no. MF501-01; Mei5 Biotechnology Co., Ltd.) were added dropwise, incubated for 30 min at room temperature and then rinsed 5 times with PBS buffer. Streptavidin-POD working solution was added according to the immunohistochemistry kit instructions, kept for 30 min and rinsed 5 times with PBS buffer. The reaction was terminated by dropwise addition of ready-to-use DAB, color development for 30 min and rinsing with distilled water. Finally, after light re-staining with hematoxylin and dehydration, neutral gum was used to seal the sections.

Immunofluorescence staining. The HK-2 cells on coverslips were fixed with 4% paraformaldehyde for 10 min at 4˚C. The cells were stained with primary antibodies against NOD-like receptor thermal protein domain associated protein 3 (NLRP3; 1:100; cat. no. ab260017; Abcam), p-NF-κB p65 (1:100; cat. no. sc-136548; Santa Cruz Biotechnology, Inc.) and secondary antibody goat anti-mouse IgG/Alexa Fluor 488 (1:1,000; cat. no. K0031G-AF488; Solarbio Technology Company, Beijing, China), respectively. Subsequently, the cells were incubated with DAPI (1 μg/ml; cat. no. C0060; Beijing Solarbio Science & Technology Co., Ltd.) for 5 min protected from light and sealed with 80% glycerol. A fluorescent inverted microscope was used to capture final fluorescence images.

Western blotting. Radioimmunoprecipitation lysis buffer was used to extract proteins from the kidney tissue and cultured cells. The concentration of protein was determined using a BCA Protein Assay Kit (cat. no. MF071-01; Mei5 Biotechnology, Co., Ltd.). Each lane was loaded with 100-120 μg of protein. Electrophoresis was performed with 10 or 15% SDS-PAGE gels. The proteins were transferred from the gels onto PVDF membranes (cat. no. IPFL00010; Merck KGaA) and blocked with a BSA-based western blot blocking solution (cat. no. MF432-01; Mei5 Biotechnology, Co., Ltd.) for 1 h at room temperature. After blocking, the PVDF membranes were incubated with primary antibodies for 2 h at room temperature and secondary antibodies for 1 h at room temperature (23). In the present study, primary antibodies against β-actin (1:10,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.), IL-1β (1:1,000; cat. no. sc-52012; Santa Cruz Biotechnology, Inc.), IL-6 (1:1,000; cat. no. sc-130326; Santa Cruz Biotechnology, Inc.), TNF-α (1:1,000; cat. no. sc-52746; Santa Cruz Biotechnology, Inc.), NF-κB p65 (1:1,000; cat. no. sc-8008; Santa Cruz Biotechnology, Inc.), phosphorylated (p-)NF-κB p65 (1:1,000; cat. no. sc-136548; Santa Cruz Biotechnology, Inc.), NLRP3 (1:1,000; cat. no. ab260017; Abcam) and monocyte chemotactic protein 1 (MCP-1; 1:1,000; cat. no. sc-52701; Santa Cruz Biotechnology, Inc.) and secondary antibodies against goat anti-rabbit IgG (1:10,000; cat. no. MF094-01; Mei5 Biotechnology, Co., Ltd.), goat anti-mouse IgG (1:10,000; cat. no. MF093-01; Mei5 Biotechnology, Co., Ltd.) and goat anti-rat IgG (1:10,000; cat. no. MF756-01; Mei5 Biotechnology, Co., Ltd.) were used for incubation. An iBright CL1000 gel imaging system program (Thermo Fisher Scientific, Inc.) was used for capturing signals, which were quantified by ImageJ software (ImageJ bundled with 64-bit Java; version 1.8.0_172; National Institutes of Health).

RNA extraction and RT-qPCR. To extract RNA, HK-2 cells were seeded in 6-well culture plates at a density of $2x10^6$ cells/well. The total RNA from HK-2 and renal tissues was isolated (Universal RNA Mini kit; Mei5 Biotechnology, Co., Ltd.) and reversed-transcribed into first strand cDNA (RevertAid First Strand cDNA Synthesis kit; Mei5 Biotechnology, Co., Ltd.), both according to the manufacturer's protocol. Subsequently, the UltraSYBR Green Mixture qPCR kit (Mei5 Biotechnology, Co., Ltd.) was used to perform RT-qPCR according to the manufacturer's protocol. The thermocycler conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 65˚C for 15 sec and 72˚C for 45 sec, ending with 1 cycle of 72˚C for 10 min. All experiments were repeated three times. The results were analyzed by the $2^{-\Delta\Delta Cq}$ method and normalized to the expression levels of the internal control gene, β-actin (24). All primer sequences used in this study are shown in Table I.

ELISA. A total of 100 μl each of cell culture medium and kidney protein suspension after drug treatment was added to a 96-well plate. The concentrations of IL-1β (HiPer Human

Table I. Specific primers for reverse transcription-quantitative PCR.

Animal	GenePrimer	Sequence (5'-3')
Mouse	NLRP3	F: ATTACCCGCCCGAGAAAGG
		R: TCGCAGCAAAGATCCACACAG
	TNF-α	F: CATGAGCACAGAAAGCATGATCCG
		R: AAGCAGGAATGAGAAGAGGCTGAG
	IL-6	F: CTGCAAGAGACTTCCATCCAG
		R: AGTGGTATAGACAGGTCTGTTGG
	IL-1β	F: CTTCAGGCAGGCAGTATCACTCAT
		R: TCTAATGGGAACGTCACACACCAG
	MCP-1	F: TAAAAACCTGGATCGGAACCAAA
		R: GCATTAGCTTCAGATTTACGGGT
	β-actin	F: ACCCTAAGGCCAACCGTGAAAAG
		R: CATGAGGTAGTCTGTCAGGT
Human	NLRP3	F: CGTGAGTCCCATTAAGATGGAGT
		R: CCCGACAGTGGATATAGAACAGA
	TNF-α	F: GAGGCCAAGCCCTGGTATG
		R: CGGGCCGATTGATCTCAGC
	IL-6	F: ACTCACCTCTTCAGAACGAATTG
		R: CCATCTTTGGAAGGTTCAGGTTG
	IL-1β	F: ATGATGGCTTATTACAGTGGCAA
		R: GTCGGAGATTCGTAGCTGGA
	MCP-1	F: CAGCCAGATGCAATCAATGCC
		R: TGGAATCCTGAACCCACTTCT
	β-actin	F: AGGCATCCTCACCCTGAAGTA
		R: CACACGCAGCTCATTGTAGA

F, forward; R, reverse.

IL-1β ELISA Kit; cat. no. MFH-02-01; Mei5 Biotechnology Co., Ltd.) and IL-18 (HiPer Human IL-18 ELISA Kit; cat. no. MFH-22-01; Mei5 Biotechnology Co., Ltd.) were detected with Elisa kit, according to the manufacturer's instructions. Then, the absorbance was measured in an enzyme marker instrument (Bioteke Corporation). The concentrations were calculated using a standard curve.

Statistical analysis. All results were evaluated using the GraphPad Prism version 8.0 (Dotmatics) and quantitative data are expressed as the mean ± standard error of the mean. One-way ANOVA and Dunnett's test for multiple comparisons were used for analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Targets of DIS in renal interstitial fibrosis. According to a network pharmacology analysis (Fig. S1), it was demonstrated that the NF-κB signaling pathway is an important pathway for DIS in improving renal interstitial fibrosis.

DIS attenuates renal pathological damage in UUO mice. The sham-operated group demonstrated no abnormal pathological changes in morphological structure (Fig. 1). In the UUO group, the renal tubular structure was disorganized, some of the tubular epithelial cells were detached from the brush border, cellular debris was occasionally seen in the tubular lumen and the renal interstitium was infiltrated with inflammatory cells and collagen was deposited. Although the ratio of urine protein to urine creatinine in mice did not show a statistically significant difference (Fig. S2), the degree of kidney tissue damage in the DIS group at each time point was less than that in the UUO group. In brief, DIS attenuated renal injury in UUO mice.

DIS attenuates the renal inflammatory response and fibrosis in the UUO mice. To further clarify the mechanism of DIS, the kidney tissue in mice was analyzed. Immunohistochemistry staining (Fig. 2) showed that the expression of TNF-α, fibronectin (FN) and α-SMA increased in the UUO group. However, the expression of TNF-α, FN and α-SMA decreased in the UUO + DIS group. Western blotting (Fig. 3A) and RT-qPCR (Fig. 3B) indicated that the expression of MCP-1, TNF-α, the ratio of p-NF-κB p65 and NF-κB p65, NLRP3, IL-1β and IL-6 increased in the UUO group compared to the sham-operated group. After being treated with DIS, the expressions of NLRP3, MCP-1, TNF-α, the ratio of p-NF-κB p65 and NF-κB p65, IL-1β and IL-6 were significantly decreased in the DIS group. Therefore, it was hypothesized that DIS alleviated renal

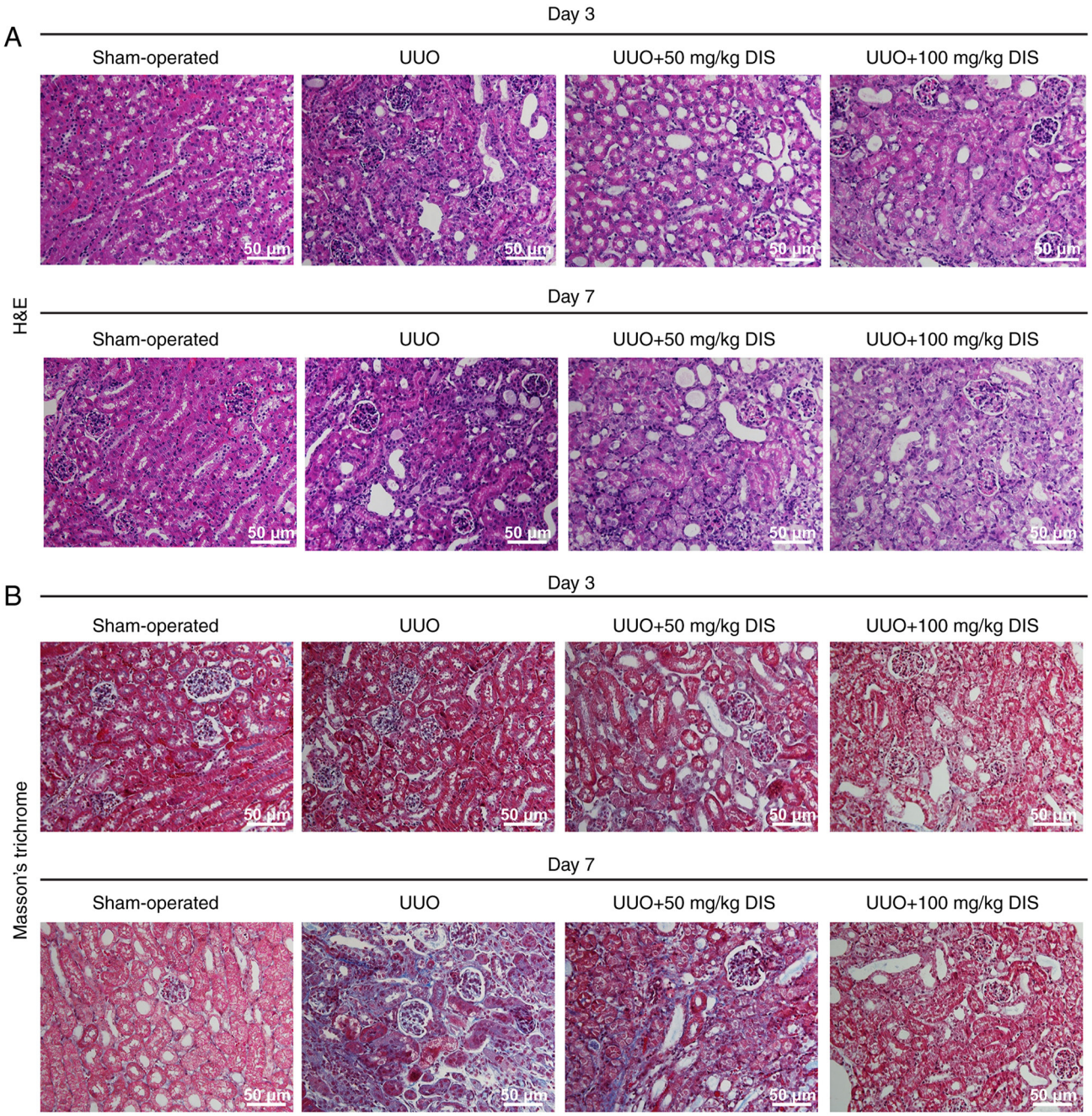

Figure 1. The effect of DIS in mice. (A) H&E staining reflected the extent of kidney damage in mice after 3 and 7 days of DIS treatment. (B) Masson's trichrome staining showed the extent of collagen fiber deposition in mice after 3 and 7 days of DIS treatment (Magnification, x200; scale bar, 50 μm). DIS, dioscin; UUO, unilateral ureteral obstruction; H&E, hematoxylin and eosin.

injury in UUO mice by inhibiting the secretion of inflammatory factors linked to the NF-κB pathway.

Effects of DIS and Bay11-7082 on TGF-β1-induced HK-2 cells. To further clarify the mechanism of DIS, TGF-β1 was used to induce a renal fibrosis model *in vitro*. First, the cells were treated with NF-κB inhibitor Bay11-7802. The expressions of NLRP3, IL-6, TNF-α, the ratio of p-NF-κB p65 and NF-κB p65, MCP-1, IL-1β and IL-18 were reduced (Fig. 4), which indicated that the inflammatory response was attenuated and the NF-κB pathway was inhibited. Subsequently, the cells were treated with DIS. The expressions of NLRP3, MCP-1, IL-6, TNF-α, IL-1β, IL-18 and the ratio of p-NF-κB p65 and NF-κB p65 were also reduced (Fig. 5), showing a similar effect to Bay11-7082, which indicated that DIS attenuated the inflammatory response by suppressing the NF-κB pathway.

Safety of DIS. To clarify the impact of DIS on renal interstitial fibrosis, the toxicity of DIS on cells was tested by CCK-8 assay. The result showed no significant toxicity to cells when the concentration of DIS was in the range of 0-20 μM and significant toxicity to cells when its concentration was in the range of 40-80 μM (Fig. 6A). The concentrations of 3.125, 6.25 and 12.5 μM were selected for further experiments and did not find that DIS trigger inflammation (Fig. 6B).

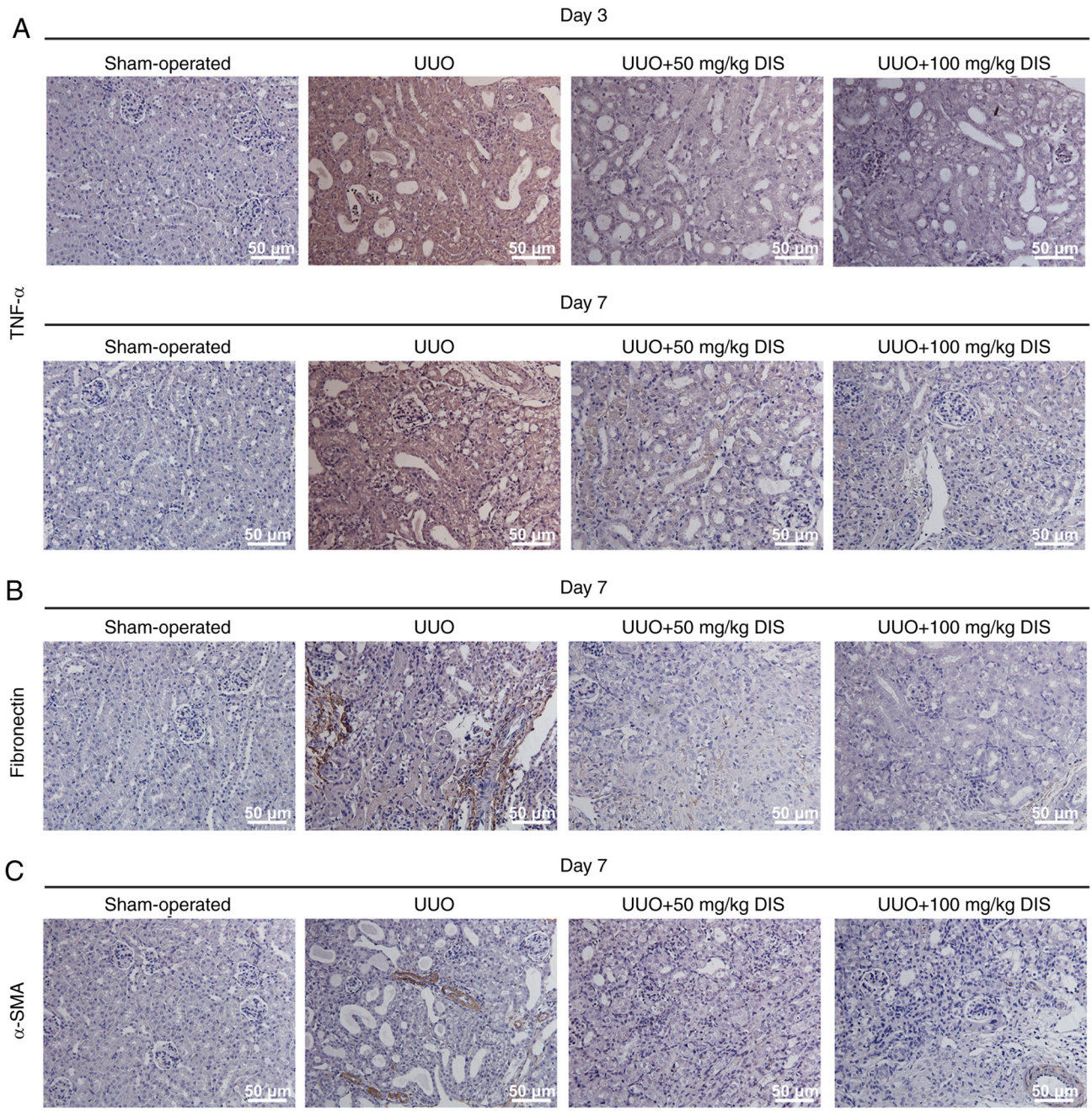

Figure 2. The effect of DIS on renal inflammation and fibrosis in mice. (A) Immunohistochemistry staining showed the expression of TNF-α after 3 and 7 days of DIS treatment. (B) Immunohistochemistry staining showed the expression of fibronectin after 7 days of DIS treatment. (C) Immunohistochemistry staining showed the expression of α-SMA after 7 days of DIS treatment (Magnification, x200; scale bar, 50 μm). DIS, dioscin; UUO, unilateral ureteral obstruction; α-SMA, α-smooth muscle actin.

Discussion

CKD has developed into a global public health issue that imposes a heavy economic burden on society and individual patients. The hallmark pathological features of CKD include interstitial fibrosis, tubular atrophy and interstitial inflammation (25). The close link between inflammation and fibrosis has become increasingly recognized (26). The inflammatory response plays a core position in the pathological mechanisms of renal fibrosis. In the early stages of CKD, various injuries induce an inflammatory response in the kidney. Persistent inflammation and cytokine release can activate fibroblasts. Activated fibroblasts overexpress α-SMA and FN, which are subsequently deposited in the kidney and trigger renal fibrosis (27). Previous studies have shown that a number of herbal extracts can reduce renal fibrosis by inhibiting the inflammatory response. Li *et al* (5) demonstrated that salidroside treatment markedly decreases the secretion of inflammatory cytokines and ameliorates renal fibrosis by the inhibition of the TLR4/NF-κB and MAPK pathway in renal interstitial fibrosis *in vivo* and *in vitro*. Similarly, Liao *et al* (28) found that isoliquiritigenin reduces renal inflammation and fibrosis in UUO-induced chronic kidney damage via inhibiting the inflammatory response and fibrosis transformation in macrophages *in vitro*. DIS is a

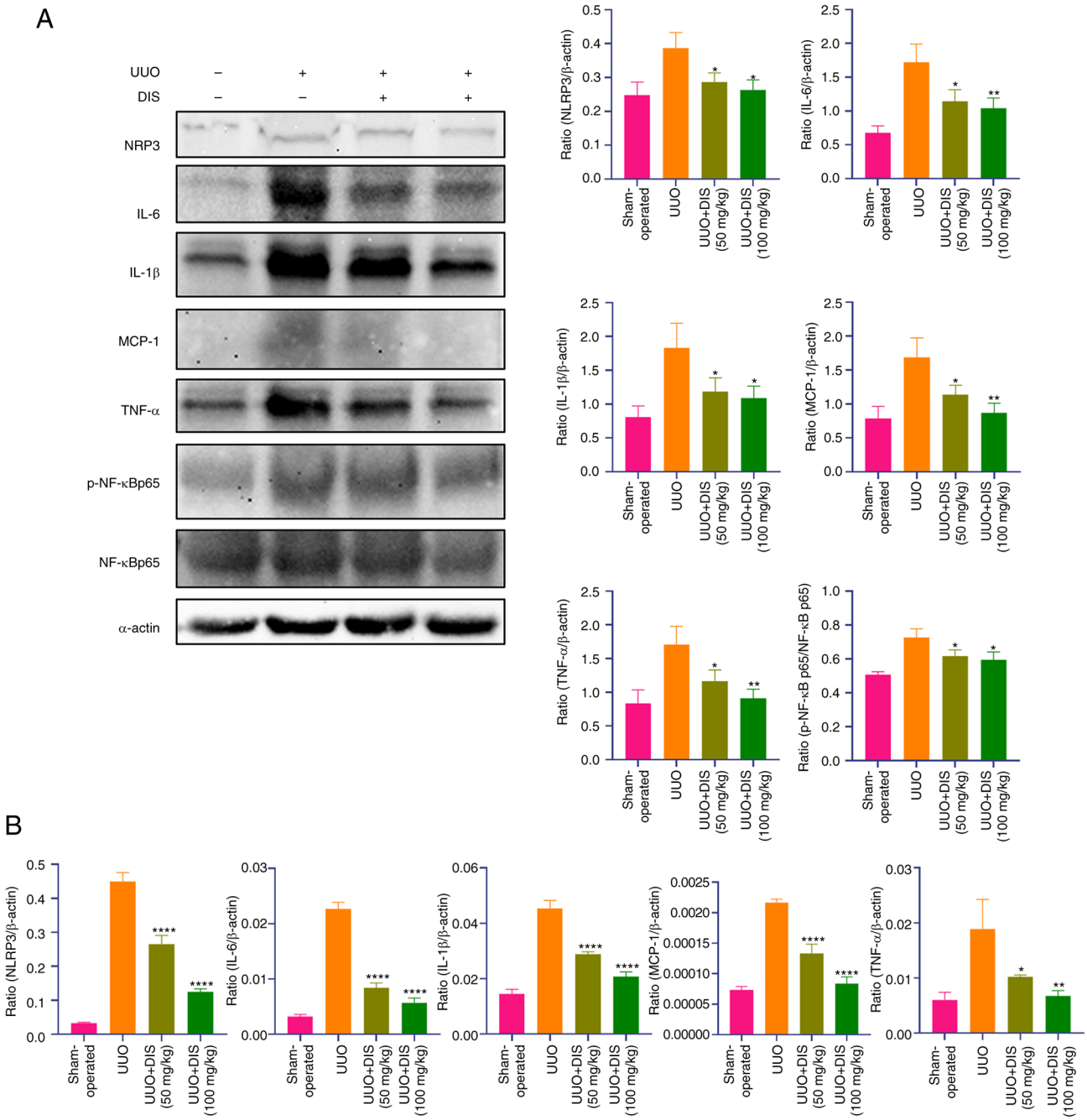

Figure 3. The effect of DIS on renal inflammation and fibrosis in mice. (A) Western blotting showed the expression of NLRP3, IL-6, IL-1β, MCP-1, TNF-α, NF-κB p65 and p-NF-κB p65 after 7 days of DIS treatment. (B) The mRNA level of NLRP3, IL-6, IL-1β, MCP-1 and TNF-α after 7 days of DIS treatment. Date represented the mean ± standard error of the mean (n=3). *P<0.05, **P<0.01 and ****P<0.0001 vs. the UUO group. DIS, dioscin; NLRP3, NOD-like receptor thermal protein domain associated protein 3; MCP-1, monocyte chemotactic protein 1; p-, phosphorylated; UUO, unilateral ureteral obstruction.

natural herbal extract with a wide range of biological activities (11). Available studies have demonstrated that DIS has a protective effect against renal injury induced by diabetic and nephrotoxic drugs, mainly through the regulation of oxidative stress, inflammatory response, lipid metabolism and cellular autophagy (17,29-32). However, no studies have been reported, to the best of the authors' knowledge, on the ability of DIS to attenuate renal fibrosis by inhibiting NF-κB signaling pathway-mediated inflammatory responses.

The NF-κB family has important roles in inflammatory responses, immune-related diseases and tumorigenesis (33). In mammals, the NF-κB family contains five-member proteins, including p65/RelA, RelB, cRel, p50 and p52 (34). Among them, NF-κB p65 is a central regulator of the inflammatory response (16). NF-κB p65 is activated by phosphorylation into p-NF-κB p65. The p-NF-κB p65 enters the nucleus and is involved in the regulation of cytokine expression, resulting in increased expressions of IL-6 (35), TNF-α (36), MCP-1, NLRP3, cytokine IL-18 and IL-1β (37). Subsequently, these cytokines participate in the inflammatory response and fibrotic processes (38).

MCP-1 is a member of the chemotactic cytokine family that recruits inflammatory cells to reach the site of injury. Reducing expression of MCP-1 reduces the renal

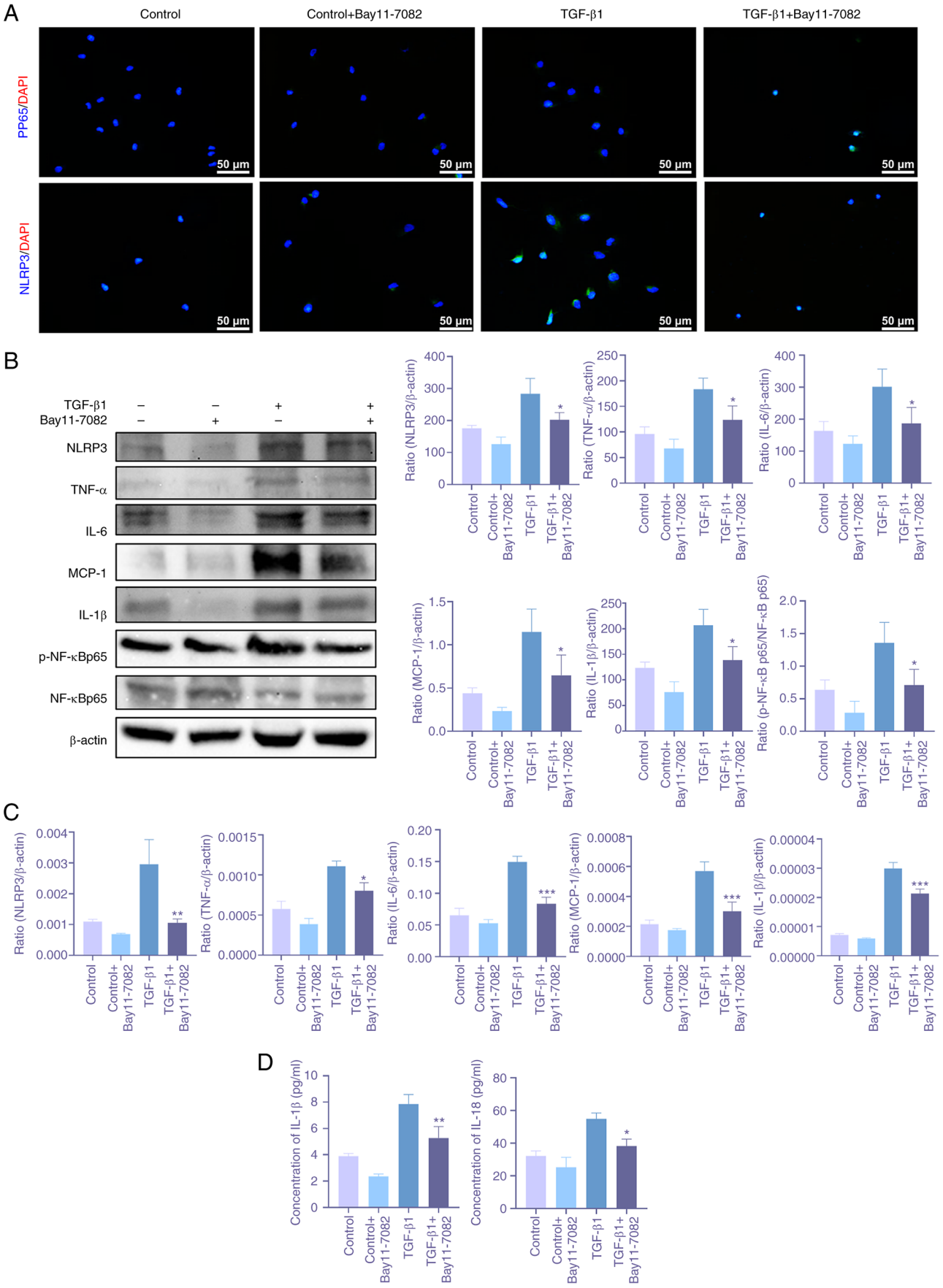

Figure 4. The effect of Bay11-7082 on renal inflammation in TGF-β induced HK-2 cells. (A) Immunofluorescence staining reflected the expressions of NLRP3 and p-NF-κB p65 (Magnification of x200; scale bar, 50 μm). (B) Western blotting showed the expressions of NLRP3, IL-6, IL-1β, MCP-1, TNF-α, NF-κB p65 and p-NF-κB p65. (C) The mRNA level of NLRP3, IL-6, IL-1β, MCP-1 and TNF-α. (D) Elisa assay reflected the concentration of IL-1β and IL-18. Date represented the mean ± standard error of the mean (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with the TGF-β1 group. NLRP3, NOD-like receptor thermal protein domain associated protein 3; MCP-1, monocyte chemotactic protein 1; p-, phosphorylated; UUO, unilateral ureteral obstruction.

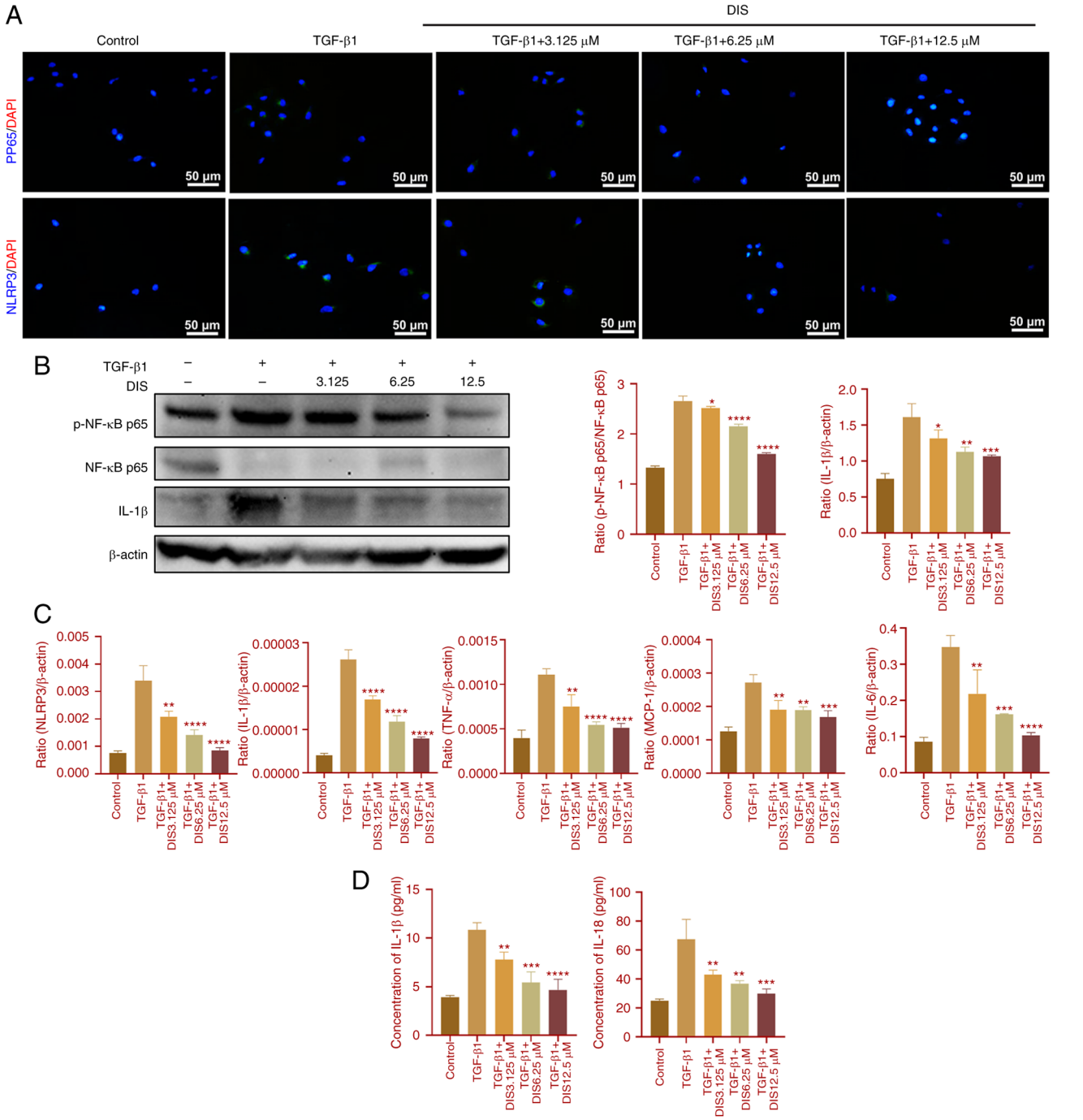

Figure 5. The effect of DIS on renal inflammation in TGF-β induced HK-2 cells. (A) Immunofluorescence staining reflected the expression of NLRP3 and p-NF-κB p65 (Magnification of x200; scale bar, 50 μm). (B) Western blotting showed the expression of IL-1β, NF-κB p65 and p-NF-κB p65. (C) The mRNA level of NLRP3, IL-1β, MCP-1, TNF-α and IL-6. (D) Elisa assay reflected the concentration of IL-1β and IL-18. Date represented the mean ± standard error of the mean (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with the TGF-β1 group. DIS, dioscin; NLRP3, NOD-like receptor thermal protein domain associated protein 3; MCP-1, monocyte chemotactic protein 1; p-, phosphorylated.

inflammatory response in mice (39). Du *et al* (40) showed that CD38 deficiency increases the phosphorylation of NF-κB p65 and the expression of its downstream target protein MCP-1 also increases in CD38-deficient wild mice. Wada *et al* (27) found that MCP-1 recruited macrophage through CCR2 signaling, which resulted in renal fibrosis. NLRP3 inflammasome is a multimeric protein complex formed by the interaction of NLRP3 and apoptosis-associated speck-like protein containing (41), which enables caspase-1 activation and thus promotes the maturation and secretion of IL-18 and IL-1β (37). It has been demonstrated that the conversion of tubular epithelial cells to renal fibrosis is modulated by NLRP3 via the TGF-β/Smad pathway in mice (42). IL-1β, a key signal in promoting fibril formation, plays a part in facilitating fibril formation by binding to IL-1βRs (43). In addition, IL-1β promotes fibrogenesis not only through c-Jun, TGF-β, PI3K/Akt and ERK1/2 but also by promoting the expression of platelet-derived growth factor receptor (43), which then binds to platelet-derived growth factor to stimulate fibrosis. This in turn promotes the proliferation and migration of

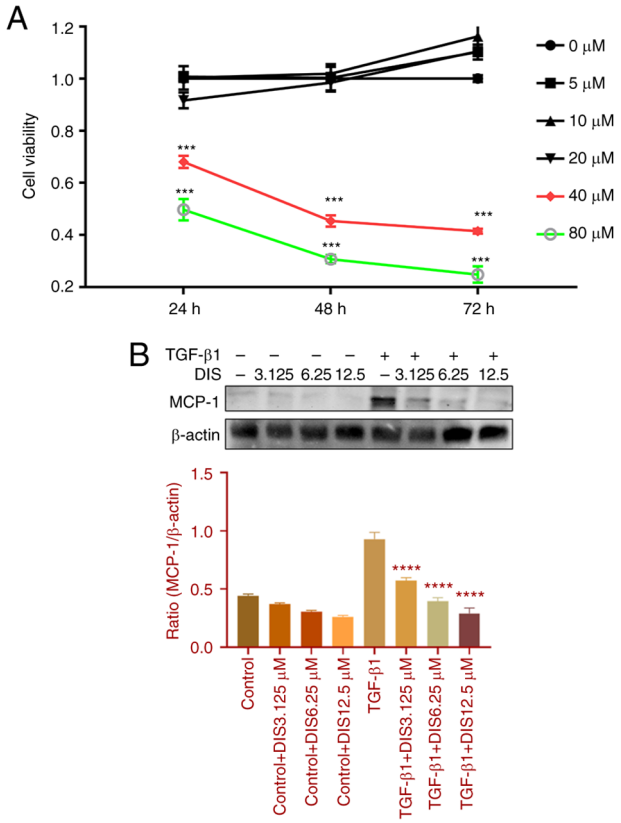

Figure 6. The efficacy and toxicity of DIS. (A) The effect of DIS on the proliferation of HK-2 cells. (B) Western blotting showed the expression of MCP-1. Date represented the mean ± standard error of the mean (n=3). ***P<0.001 and ****P<0.0001 compared with the TGF-β1 group. DIS, dioscin; MCP-1, monocyte chemotactic protein 1.

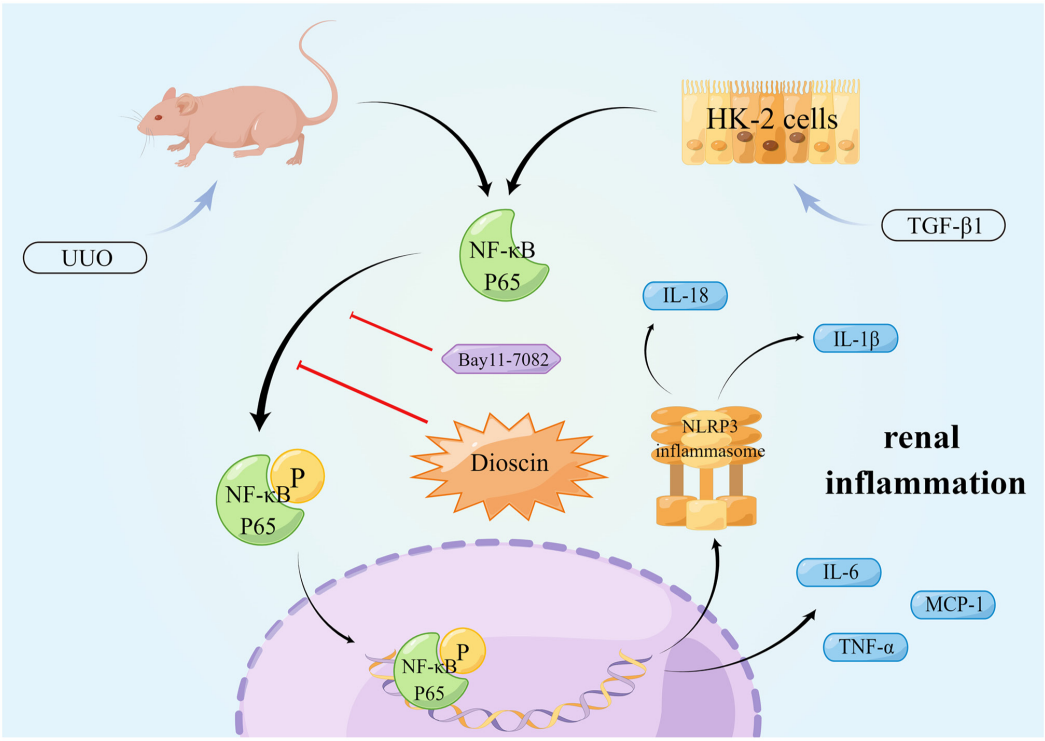

Figure 7. An overview of the potential mechanism by which dioscin (DIS) inhibits the NF-κB signaling pathway to reduce renal inflammation and delay renal fibrosis. DIS treatment inhibits the phosphorylation of NF-κB p65, downregulates NLRP3 inflammasome expression and thereby reduces IL-1β secretion, as well as IL-6, TNF-α, MCP-1 and IL-18 secretion, ultimately reducing renal inflammation in UUO mice and TGF-β1-stimulated HK-2 cells. Meanwhile, Bay11-7082, an inhibitor of NF-κB p65 phosphorylation, was able to reduce renal inflammation by inhibiting NF-κB p65 phosphorylation, down-regulating NLRP3 inflammasome expression and ultimately attenuating the secretion of IL-1β, IL-6, TNF-α, MCP-1 and IL-18. DIS, dioscin; NLRP3, NOD-like receptor thermal protein domain associated protein 3; MCP-1, monocyte chemotactic protein 1; UUO, unilateral ureteral obstruction.

renal stromal cells, leading to the deposition of ECM and the formation of renal interstitial fibrosis (44). IL-18 also has a pro-fibrotic effect by promoting IFNγ and IL-13 production in Th1 cells (45) and inducing macrophage differentiation into myofibroblast (46). In addition, Zhao *et al* (47) found that the activation of NLRP3 inflammasome could be regulated by phosphorylation of NF-κB p65. IL-6 is a classical cytokine that plays an important part in inflammation, autoimmune diseases, fibrotic diseases and cancer. IL-6 gene expression is regulated by the NF-κB pathway (35). A previous study found that TNF-α, mainly in macrophages, renal tubular cells and thylakoid cells, can trigger an inflammatory response and renal fibrosis (48). This study also found that renal fibrosis injury in mice was significantly reduced after treatment with a TNF receptor-1 inhibitor.

The present study (Fig. 7) established a renal fibrosis model in mice by UUO. In the hematoxylin and eosin and Masson's trichrome staining, it was observed that the interstitial structure of the kidney in the UUO group was disturbed, accompanied by massive fibrotic deposits and inflammatory cell infiltration. However, in the DIS group, inflammatory cell infiltration in renal tissue was alleviated and collagen fiber deposition was reduced. In addition, the expression of TNF-α, FN and α-SMA were reduced. Therefore, it can be concluded that DIS has a protective effect on the kidney. In addition, in western blotting and RT-qPCR, the expressions of IL-1β, IL-6, NLRP3, TNF-α and MCP-1 and the phosphorylation NF-κB p65 were downregulated in the DIS group. In summary, the present study found that DIS inhibited the inflammatory response in mice and also attenuated renal fibrosis, so it was hypothesized that this is related to the inhibition of the NF-κB signaling pathway. To further clarify the mechanism of the renal protective effect of DIS, a renal fibrosis model was established by using TGF-β-induced HK-2 cells *in vitro*. First, the cells were treated with Bay11-7082, a known inhibitor of NF-κB nuclear transcription factor (49), which inhibits the phosphorylation of nuclear transcription factor NF-κB p65 and regulates the expression of downstream genes. It was shown that the expressions of inflammatory mediators including NLRP3, TNF-α, IL-1β, IL-6, MCP-1 and IL-18 were downregulated in cells treated with Bay11-7082. In addition, the ratio of p-NF-κB p65 and NF-κB p65 was also reduced. Similarly, the expressions of inflammatory mediators as well as the ratio of p-NF-κB p65 and NF-κB p65 were downregulated under the DIS treatment.

In conclusion, the present study found that DIS attenuated the inflammatory response and renal interstitial fibrosis and that the mechanism may be the suppression of NF-κB pathway-mediated inflammatory response.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW, PeL, PiL and NL conceived and designed the study. YW, GM, CW and WZ conducted experiments on the cells and the data analysis, and wrote part of the original manuscript. PS, WL, XY and YZ conducted experiments on the mice and the data analysis, and wrote part of the original manuscript. YW, NL and PiL reviewed and edited the manuscript. PeL, NL and PiL participated in the funding acquisition and confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Ethics Committee of the China-Japan Friendship Institute of Clinical Medical Sciences (approval no. zryhyy21-22-01-09; affiliated with the China-Japan Friendship hospital, Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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