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Dihydroartemisinin attenuates renal fibrosis through regulation of fibroblast proliferation and differentiation

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ABSTRACT

Aims: Renal fibrosis is the most common final stage of progressive renal disease, characterized by fibroblast proliferation, fibroblast-to-myofibroblast differentiation and excessive accumulation of extracellular matrix. Dihydroartemisinin (DHA) exerts antitumor, antibacterial, and antifibrotic effects. The aim of this study was to determine whether DHA has beneficial effects on unilateral ureteral obstruction (UUO)-induced renal fibrosis in mice and to examine explore the underlying possible mechanisms.

Materials and methods: Eight-week-old male C57BL/6 mice were intragastrically administered DHA for 14 consecutive days after UUO operation. Afterward, interstitial collagen deposition, expression of collagen I and III, fibronectin, α-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA), and S100 calcium-binding protein A4 (S100A4) were assessed in the kidneys. Transforming growth factor beta 1 (TGF-β1)-induced primary human kidney fibroblasts were treated with DHA to further investigate the mechanism underlying its action.

Key findings: In vivo, DHA reduced UUO-induced morphological and pathological changes and the degree of renal fibrosis. In addition, DHA mitigated fibroblast proliferation and differentiation in kidney tissue induced by UUO. In vitro, DHA significantly attenuated the TGF-β1-induced primary human kidney fibroblast proliferation and fibroblast-to-myofibroblast differentiation. Moreover, treatment with DHA attenuated the up-regulation of phosphorylation of phosphatidylinositol-3-kinase (PI3K) and protein kinase B (AKT) in UUO model and TGF-β1-induced primary human kidney fibroblasts.

Significance: We provide in vivo and in vitro evidence that DHA may relieve renal fibrosis through regulation of fibroblast proliferation and differentiation by mitigating the PI3K/AKT pathway. DHA may potentially be used as a therapeutic antifibrotic agent for the treatment of renal fibrosis.

1. Introduction

Renal fibrosis is a common pathological process occurring in chronic kidney disease (CKD), characterized by thickening of the tubular basement membrane and excessive accumulation of extracellular matrix (ECM). Renal fibrosis is caused by a complex set of etiological factors, such as inflammatory, immunological, metabolic, obstructive, or other systemic diseases [1,2]. Numerous studies have revealed that renal fibrosis is mediated by multiple mechanisms, and the central cellular event is the proliferation of fibroblasts and their differentiation into myofibroblasts, induced by various factors [3]. Myofibroblasts, which are characterized by the development of α-smooth muscle actin (α-SMA) fibers, have a contractile phenotype and abundantly synthesize collagen and ECM proteins [4]. Persistent production of ECM decreases the glomerular filtration rate and adversely affects renal function, eventually resulting in the acceleration of renal injury [5]. However, there are no effective therapies to completely prevent the progression of renal fibrosis or slow the progressive loss of renal function in CKD. The treatment options for patients with end-stage renal disease are limited to dialysis and renal transplantation.

Abbreviations: DHA, dihydroartemisinin; UUO, unilateral ureteral obstruction; ECM, extracellular matrix; CKD, chronic kidney disease; BUN, blood urea nitrogen; Scr, serum creatinine; CT, computed tomography; α-SMA, α-smooth muscle actin; S100A4, S100 calcium-binding protein A4; PCNA, proliferating cell nuclear antigen; TGF-β1, transforming growth factor beta 1; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; CCK-8, Cell Counting Kit-8; H&E, Hematoxylin and Eosin; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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Meanwhile, herbal medicines have traditionally been used for CKD, and their constituent natural compounds are considered essential sources for drug discovery [6].

Dihydroartemisinin (DHA), derived from the natural small-molecule compound artemisinin, is a safe and effective antimalarial drug recommended by the World Health Organization [7]. In recent years, DHA has attracted attention of researchers because it exhibits an ample array of pharmacological activities, such as antitumor, antibacterial, and antifibrotic properties [8–10]. A research showed that DHA significantly decreased collagen synthesis and inhibited fibroblast proliferation in bleomycin-induced pulmonary fibrosis [11]. Another research demonstrated that DHA alleviated oxidative stress and reduced the increase in the myofibroblasts-like processes of the type II alveolar epithelial cells (AECs) in bleomycin-induced pulmonary fibrosis [12]. Thus, we hypothesize that DHA may prevent renal fibrosis and try to explore the effect of DHA on fibroblast proliferation and myofibroblasts activation and its potential mechanism.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (also known as AKT) pathway plays a significant role during the process of fibrosis by regulating many upstream and downstream factors and can promote the proliferation of fibroblasts and their differentiation into myofibroblasts [13]. Some studies have shown that natural compounds could regulate the fibrosis-related PI3K/AKT pathway and indicated that PI3K/AKT signaling might be a potential target for antifibrosis therapy [14,15].

The aims of the present study were to investigate whether DHA attenuates renal fibrosis and to explore the underlying mechanism. In this study, we determined the in vivo and in vitro protective effects of DHA against renal fibrosis using a unilateral ureteral obstruction (UUO) model and a transforming growth factor beta 1 (TGF-β1)-stimulated primary human kidney fibroblast model, respectively. Our results confirmed that DHA attenuated UUO-induced kidney fibrosis, and also prevented the fibroblast proliferation and fibroblast-to-myofibroblast differentiation partly through regulation of PI3K/AKT pathway.

2. Materials and methods

2.1. Drug

DHA (> 98% purity; MW 284.35) was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

2.2. Ethics statement

The protocols of the experiments were approved by the Committee on the Ethics of Animal Experiments of Central South University (China). All experiments were performed in accordance with the guidelines of the National Institutes of Health. The mice were anesthetized by intraperitoneal injection with chloral hydrate (400 mg/kg), and all efforts were made to minimize animal suffering while performing surgery.

2.3. Animals and treatments

Animal experiments were conducted on 8-week-old male C57BL/6 mice of specific-pathogen-free grade (Department of Laboratory Animal Unit of Central South University, China). The animals were housed under a 12/12-h light/dark cycle and controlled temperature (23 ± 1 °C). The method used for the UUO operation was as described in a previously published study [16]. Briefly, all surgeries were performed under anesthesia with 10% chloral hydrate (400 mg/kg). In the UUO group, the left lateral dorsal surface of the mice was incised, the left ureter was exposed and ligated with 4-0 silk sutures, and the ligatures were cut off to prevent retrograde urinary tract infections. The sham operation was performed in a similar manner, except for the ureter ligation.

Twenty-four male C57BL/6 mice were randomly divided into the following four groups: (1) a sham group; (2) a UUO group; (3) a DHA group; and (4) a UUO + DHA group. The mice in the latter two groups were intragastrically administered DHA (40 mg/kg once a day) for 14 consecutive days after surgery. The mice in the sham and UUO groups were administered equal volumes of a 0.9% NaCl solution. All animals were sacrificed on day 14 after surgery. The left kidneys were decapsulated, washed with ice-cold normal saline, and then rapidly dissected. Part of each kidney was fixed in 10% formalin, and the remaining part was stored at −80 °C for further study.

2.4. Renal function analysis

All animals were sacrificed on the 14th day after surgery. Blood was taken from the ophthalmic artery, and the serum obtained by centrifugation was immediately sent to the department of Clinical Laboratory, Xiangya Hospital, Central South University for determination of blood urea nitrogen (BUN) and serum creatinine (Scr) levels by colorimetry assays (Beckman AU5800, USA).

2.5. Histological examination

Kidney tissues were fixed in a 4% paraformaldehyde solution and then embedded in paraffin for the preparation of tissue sections for pathological examination, for which the sections were stained with hematoxylin and eosin (H&E), Masson's trichrome, and Sirius red. For H&E staining, slides were immersed in a hematoxylin solution for 3 to 5 min, then differentiated with acid alcohol, and counterstained with eosin for 3 min. For Masson's trichrome staining, slides were immersed in a potassium bichromate solution overnight and then sequentially stained with a mixed hematoxylin A and B solution for 3 min, a Ponceau–acid fuchsin solution for 5 min, a phosphomolybdic acid solution for 2 min, and an aniline solution for 5 min. For Sirius red staining, slides were immersed in a Sirius red solution for 10 min. Images of H&E- and Masson's trichrome-stained sections were acquired under a microscope (Nikon, Japan). Images of Sirius red-stained sections were acquired using polarized light microscopy (Nikon, Japan).

2.6. Immunohistochemical examination

In brief, paraffin sections were deparaffinized, rehydrated, immersed in 3% hydrogen peroxide for 10 min to inactivate any endogenous peroxidase, and then incubated for 30 min in blocking buffer containing 5% bovine serum albumin (BSA). After blocking, the sections were incubated overnight at 4 °C with antibodies against collagen I, collagen III (1:100; Servicebio, China), followed by incubation at room temperature (20–25 °C) with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:100; Sigma–Aldrich, St. Louis, MO, USA). Images of five randomly selected fields were acquired under a microscope (Nikon, Japan).

2.7. Immunofluorescence staining

Briefly, paraffin-embedded sections (5-μm thickness) were deparaffinized, and non-specific binding was blocked by incubation with 3% BSA for 30 min. Thereafter, the slides were incubated with primary antibodies against PCNA (1:100; Servicebio, China), S100A4 (1:100; Boster, China), and α-SMA (1:100; Servicebio, China) overnight at 4 °C, followed by incubation with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:100; Beyotime, China) or an Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (1:100; Beyotime, China) at room temperature for 30 min in the dark. Finally, the slides were incubated with a 4′,6-diamidino-2-phenylindole solution at room temperature for 10 min and mounted with coverslips using anti-fade medium. Images were acquired using fluorescent microscopy (Nikon, Japan).

2.8. Primary human kidney fibroblasts isolation and culture

Segments of macroscopically and histologically normal renal cortex were obtained from the kidney of a 45-year-old male that was surgically

removed as part of treatment for renal cell carcinoma (tumor stage: T2aN0M0; largest tumor diameter: 7.2 cm). The patient had no hypertension, diabetes, tuberculosis, or any other disease before surgery. No renal impairment was noted; BUN and Scr levels were 7.1 mmol/L and 82.9 μmol/L, respectively. Before surgery, a 64-multidetector row computed tomography (CT) scanner with a 0.5-mm step interval was used in the patient to acquire enhanced CT scans, including arterial, parenchymal, and secretory phases, to determine the staging of the tumor. Informed consent was obtained before each operative procedure, and the use of human renal tissue was approved by the Xiangya Hospital Human Research Ethics Committee. Briefly, normal renal tissue was isolated at least 5 cm from the edge of the tumor, renal cortical tissue was dissected from the medulla, chopped the with surgical scissors until finely minced, digested with 5 ml enzyme mix (1 mg/ml Collagenase I (Sigma-Aldrich, USA) and 10 μg/ml Dnase I (Solarbio, China), incubate at 37 °C for 1 h with gentle agitation. Added 10 ml PBS and passed the entire suspension through 80 μm cell strainer into a new 50 ml tube. Centrifuge for 5 min at 500 ×*g*. Removed the supernatant and used red blood cell lysate (Solarbio, China) to remove the red blood cell. Cells were resuspended in Dulbecco's modified Eagle's media and Ham's F-12 (DMEM/F-12) (Hyclone, USA) containing 10% fetal calf serum (FBS) (Cyagen Bioscience, China), 1% antibiotics (streptomycin and penicillin) and were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Cells were used between the second and sixth passages. To investigate the effect of DHA on the TGF-β1-induced proliferation and differentiation of fibroblast, Prior to TGF-β1 treatment, fibroblasts were quiescent by growing in DMEM/F-12 with 0.2% FBS for 12 h, then pretreated with DHA (10 μmol/L(μM), Macklin Biochemical Co. Ltd., China) for 1 h, following treatment with TGF-β1 (10 ng/ml, Peprotech, USA) for 24 h.

2.9. Cell proliferation assay

For various treatment conditions, primary human kidney fibroblasts were plated in 96-well plates and cultured with or without DHA and TGF-β1 for 24 h. Primary human kidney fibroblasts proliferation was investigated using a Cell Counting Kit 8 (Dojindo, Japan) according to the manufacturer's instructions.

2.10. Quantitative polymerase chain reaction

Total RNA was isolated from kidney tissue and primary human kidney fibroblasts using the TRIzol reagent (TaKaRa, Japan) according to the manufacturer's protocol. Reverse transcription (RT) of RNA (1 μg) to cDNA was performed using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan) in a thermal cycler (Bio-Rad, USA). The primers used for the target genes are listed in Table 1. Real-time quantitative polymerase chain reaction (qPCR) was conducted using SYBR Premix Ex Taq II (TaKaRa, Japan) in a real-time PCR detection system (CFX96 Touch™, Bio-Rad, USA). The relative expression of collagen I, collagen III, fibronectin, and α-SMA mRNA was determined by normalizing the expression level of each gene to that of the GAPDH gene using the $2^{-\Delta\Delta Ct}$ method.

2.11. Western blotting

Total protein lysates were obtained from kidney tissue and primary human kidney fibroblasts using the radioimmunoprecipitation assay buffer (Beyotime, China) with a proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and PhosSTOP(Roche Diagnostics, Indianapolis, IN, USA). The protein concentration was estimated using a bicinchoninic acid assay kit (Sigma, USA). Equivalent amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Millipore, USA). After blocking, the membranes were incubated with primary antibodies against collagen I (1:1000; Boster, China), collagen III (1:1000; Servicebio, China), fibronectin (1:1000; Servicebio, China), α-SMA (1:500; Servicebio, China), PCNA (1:2000; Servicebio, China), phospho (p)-PI3K (1:1000; Cell Signaling Technology, USA), PI3K (1:1000; Proteintech Group, China), p-AKT (1:1000; Servicebio, China), AKT (1:1000; Servicebio, China), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000; Servicebio, China) overnight at 4 °C. Subsequently, the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (1:5000 goat anti-rabbit IgG, Boster, or 1:3000 goat anti-mouse IgG, Millipore, USA). Protein bands were detected using enhanced chemiluminescent reagents (Millipore, USA). Images were obtained using a ChemiDoc XRS system (Bio-Rad, USA).

2.12. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Comparisons among different groups were made by one-way analysis of variance, followed by the Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism 6.0.

3. Results

3.1. DHA attenuated morphological changes and renal function impairment in the UUO model

H&E staining showed that in the sham and DHA groups, the glomerular and tubular structures were normal, and tubules were packed closely in renal tissue, which indicated that DHA (40 mg/kg/day) caused no obvious damage to the renal structure, therefore, 40 mg/kg DHA was used in subsequent experiments (Fig. 1A). In the UUO group, the interstitial spaces were significantly wider, with infiltration of inflammatory cells and perivascular exudates, while glomerular capillaries became dilated, and tubular cells underwent necrosis and atrophy

Table 1
The primer sequences for real-time PCR.

Gene	Forward primer	Reverse primer
h-GAPDH	CCAGAACATCATCCCTGCCT	CCTGCTTCACCACCTTCTTG
h-α-SMA	TGCCTTGGTGTGTGACAATG	TCACCCACGTAGCTGTCTTT
m-GAPDH	GAAGGTGGTGAAGCAGGCATCT	
CGGCATCGAAGGTGGAAGAGTG	m-Fibronectin	
TCCCGGGCAGAAAGTACATT	TTCAGGGAGGTTGAGCTCTG	
m-Collagen I	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
m-Collagen III	CCCCTGGTTCTTCTGGACAT	
TGGGCCTTTGATACCTGGAG	m-α-SMA	
CTTCGCTGGTGATGATGCTC	GTTGGTGATGATGCCGTGTT	

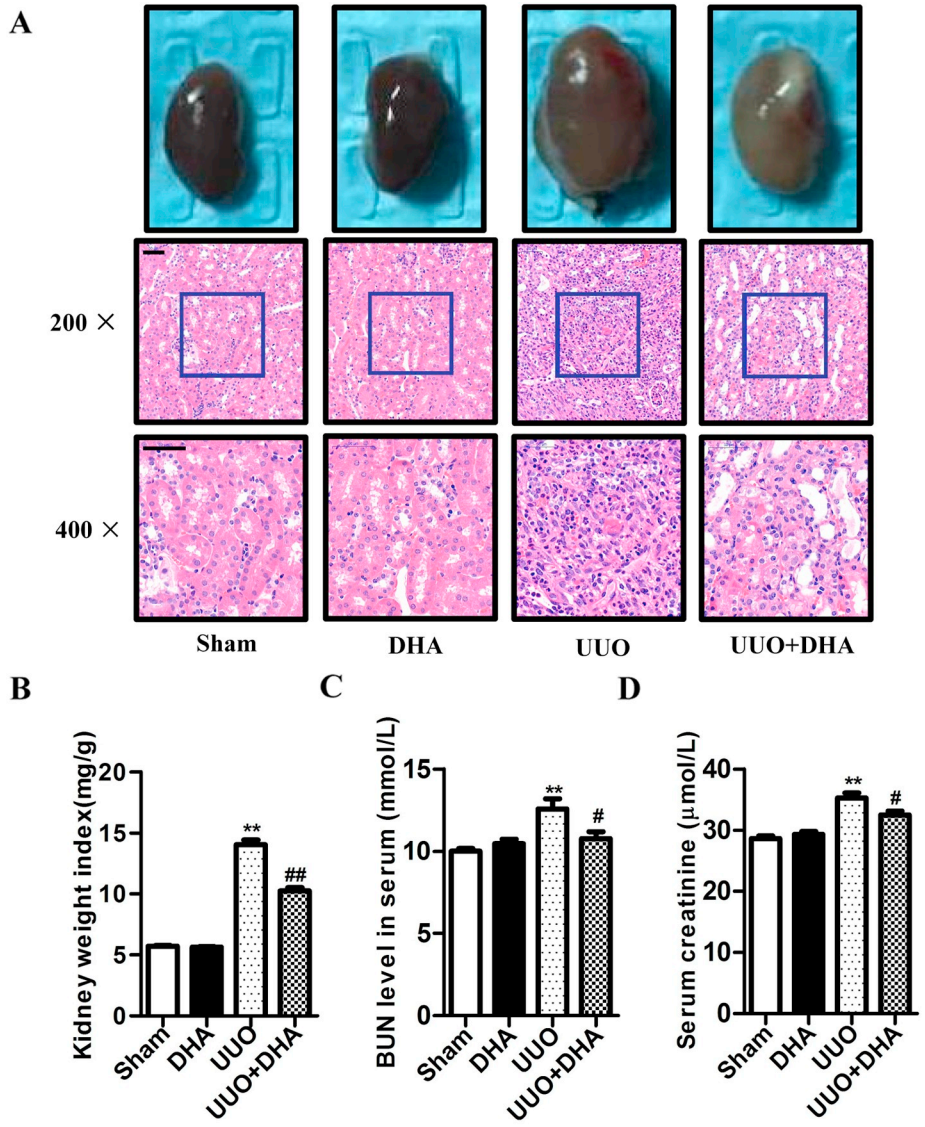

Fig. 1. Effects of DHA on morphological changes and renal function. (A) Gross histopathology of the kidney. Representative kidney sections were stained with H&E (top panel: ×200 magnification, bar = 50 μm; bottom panel: ×400 magnification, bar = 50 μm). (B) Changes of in the kidney weight/body weight ratio in mice. (C) Measurement of the levels of BUN in serum of mice. (D) Measurement of the levels of Scr in serum of mice. The data are presented as the mean ± SD, $n = 5–7$. $^{**}P < 0.01$ versus Sham group; $\#P < 0.05$, $\#\#P < 0.01$ versus UUO group.

(Fig. 1A). Meanwhile, in the UUO + DHA group, the morphological changes induced by UUO significantly improved (Fig. 1A). In addition, the kidney weight index was significantly higher in the UUO group than in the sham group. However, treatment with DHA significantly reduced the kidney weight index compared with that in the UUO group (Fig. 1B). As shown in Fig. 1C and D, the levels of BUN and Scr were significantly higher in the UUO group than in the Sham group. However, treatment with DHA at doses of 40 mg/kg/day significantly decreased Scr and BUN levels, which indicates the ability of DHA to improve renal function. These results showed that DHA prevented morphological changes and renal function impairment induced by UUO in vivo.

3.2. DHA alleviated renal collagen deposition and interstitial fibrosis in the UUO model

Extracellular matrix deposition is the significant characteristic of fibrosis, including collagens, fibronectin, elastin, laminin, and etc. [17]. As shown in Fig. 2A, the collagen deposition was not observed in the sham and DHA groups, while collagen deposition was obvious in renal tissues from the UUO group, stained with Masson's trichrome and Sirius red (Fig. 2A). Compared with those from the UUO group, renal tissues from the DHA-treated mice showed reduced collagen deposition areas (Fig. 2A). Moreover, the immunohistochemical staining results showed that the expression of collagen I and collagen III, which were the important component of extracellular matrix, markedly increased in renal tissues from the UUO group on day 14 and was significantly reduced in the UUO + DHA group (Fig. 2A).

Furthermore, our results showed that the mRNA levels of collagen I, collagen III, and fibronectin were markedly higher in the UUO group than in the sham group (Fig. 2B). In the UUO + DHA group, the mRNA levels of collagen I, collagen III, and fibronectin significantly decreased compared with those in the UUO group. We also found that the protein expression of collagen I, collagen III, and fibronectin was significantly higher in the UUO mice than in the sham group. Intervention with DHA significantly inhibited the upregulation of collagen I, collagen III, and fibronectin in UUO mice at the protein level (Fig. 2C). These data revealed that DHA attenuated extracellular matrix deposition and the extent of fibrosis induced by UUO.

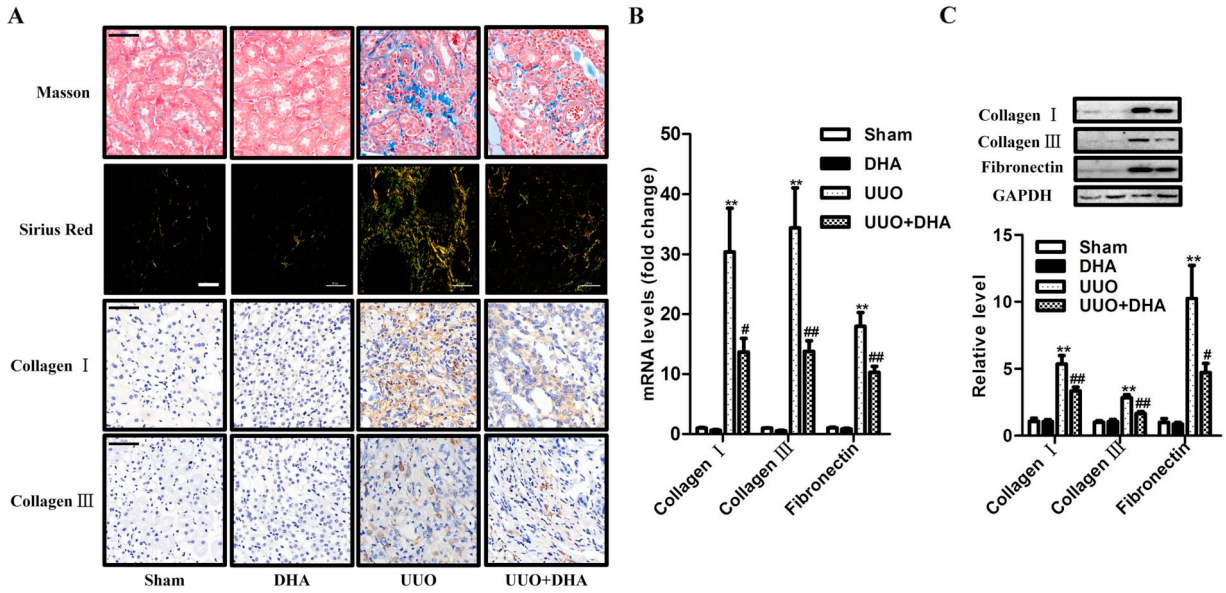

Fig. 2. Effects of DHA on ECM deposition in the UUO model. (A) Representative micrographs of Masson's trichrome- and Sirius red-stained renal tissues, and immunohistochemical staining of renal sections for collagen I and III. (B) Expression levels of collagen I, collagen III, and fibronectin mRNA in renal tissue of mice, determined by qPCR. (C) Protein expression of collagen I, collagen III, and fibronectin in renal tissue of mice. The data are presented as the mean ± SD, $n = 5–7$. $^{**}P < 0.01$ versus Sham group; $\#P < 0.05$, $\#\#P < 0.01$ versus UUO group.

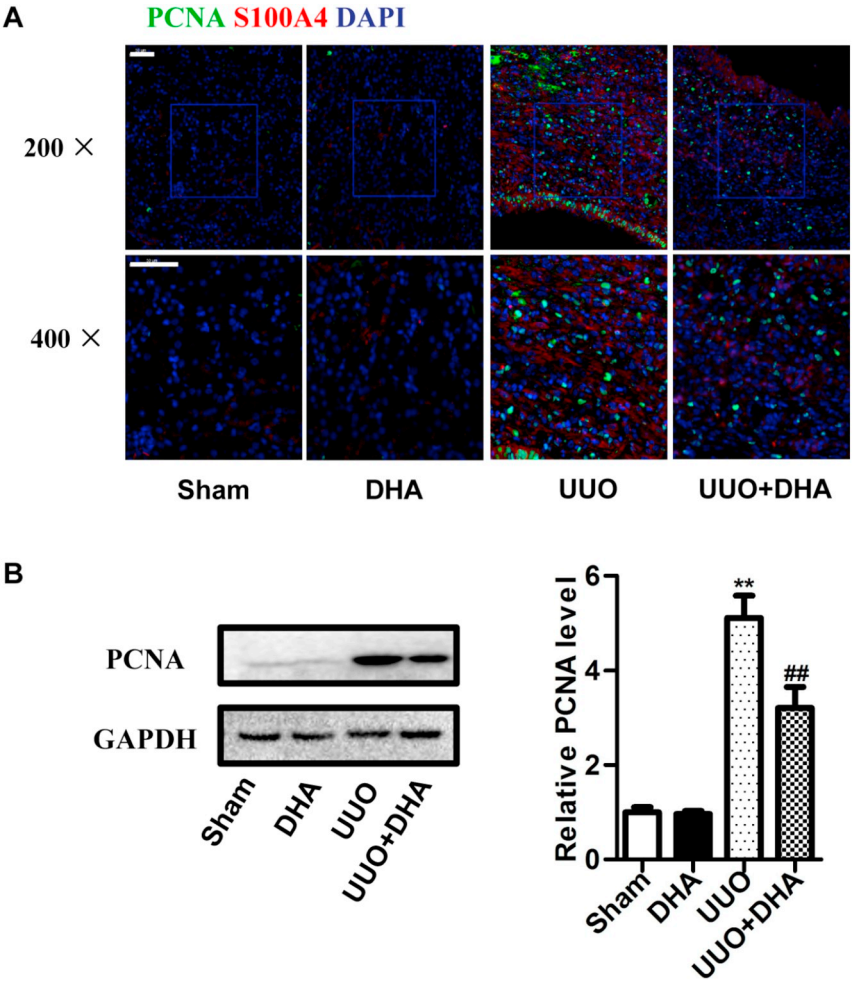

Fig. 3. Effects of DHA on the proliferation of fibroblasts in the UUO model in vivo. (A) Expression of S100A4 and PCNA in renal tissue, detected by immunofluorescence. (B) Protein expression of PCNA in renal tissue, determined by western blotting. The data are presented as the mean ± SD, $n = 3$. $^{**}P < 0.01$ versus Sham group; $\#\#P < 0.01$ versus UUO group.

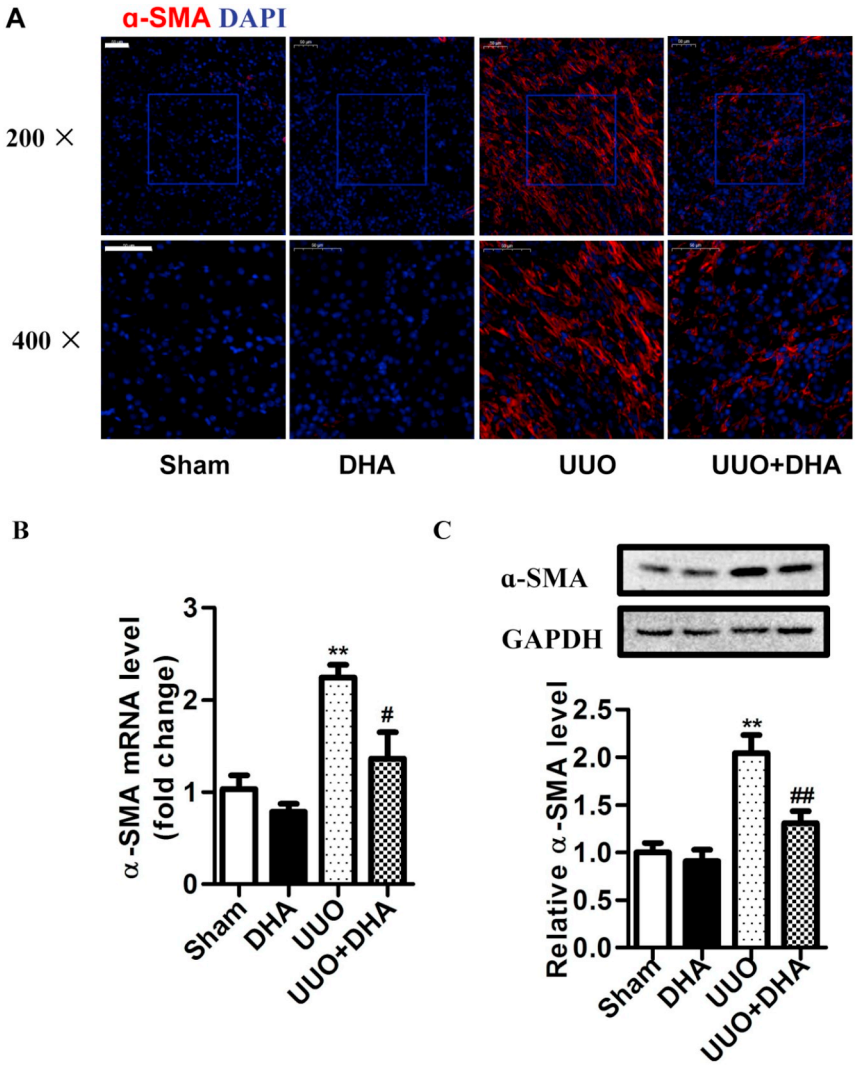

Fig. 4. Effects of DHA on the differentiation of fibroblasts in the UUO model in vivo. (A) Expression of α-SMA in renal tissue, detected by immunofluorescence. (B) mRNA levels of α-SMA were quantified by qPCR. (C) Protein expression of α-SMA in renal tissue was determined by western blotting. The data are presented as the mean ± SD, n = 3. $^{**}P < 0.01$ versus Sham group; $\#P < 0.05$, $\#\#P < 0.01$ versus UUO group.

3.3. DHA mitigated proliferation of kidney fibroblasts in the UUO model

S100A4 (also known as fibroblast-specific protein 1) has been widely accepted as a fibroblast-specific marker [18]. PCNA, which plays an important role in DNA synthesis, DNA repair, cell cycle progression, and cell proliferation, is a sensitive indicator of proliferation [19,20]. To evaluate whether DHA could mitigate fibroblast proliferation, the expression of S100A4 and PCNA was detected on postoperative day 14 in kidney tissue through immunofluorescence staining. Kidney sections showed high positive PCNA coexpressed with S100A4 in the fibroblast of UUO-induced mice but not in control and DHA groups kidney. In contrast, the number of fibroblasts (S100A4 positive) coexpressed with PCNA were significantly lower in the UUO + DHA group than those in the UUO group (Fig. 3A). In addition, significantly lower expression of PCNA in renal tissue from the UUO + DHA group compared with that of the UUO group was confirmed by western blotting (Fig. 3B). These results indicated that DHA attenuated fibroblast proliferation in vivo.

3.4. DHA mitigated myofibroblast activation in the UUO model

To further confirm the effect of DHA on myofibroblast activation, the major molecular marker of myofibroblasts, α-SMA, was detected by immunofluorescence, qPCR, and western blotting. Immunofluorescence staining (Fig. 4A) showed that α-SMA positive cells notably increased in the kidneys of UUO mice compared with that in the sham group. However, DHA treatment mitigated the number of α-SMA positive cells. Moreover, mRNA (Fig. 4B) and protein (Fig. 4C) levels of α-SMA dramatically increased in the UUO group compared with those in the sham group. qPCR and western blotting analyses indicated that the levels of α-SMA were significantly lower in the UUO + DHA group than in the UUO group. These results revealed that DHA alleviated the myofibroblast activation induced by UUO in the kidney tissue.

3.5. DHA inhibited TGF-β1-induced proliferation and differentiation of primary human kidney fibroblasts in vitro

Appearance of the isolated primary human kidney fibroblasts is shown in Fig. 5A. To determine the effect of DHA on cell viability, the primary human kidney fibroblasts were treated with different DHA concentrations (0, 3, 10, 30 μM). The CCK-8 assay showed that 30 μM DHA significantly decreased cell viability (Fig. 5B); therefore, for further experiments, we chose a lower concentration (10 μM) that had no obvious deleterious effect on the cells. TGF-β1 is the most important profibrotic factor, which is often used as the stimulator for fibroblasts proliferation and differentiation [21]. The CCK-8 assay showed that the

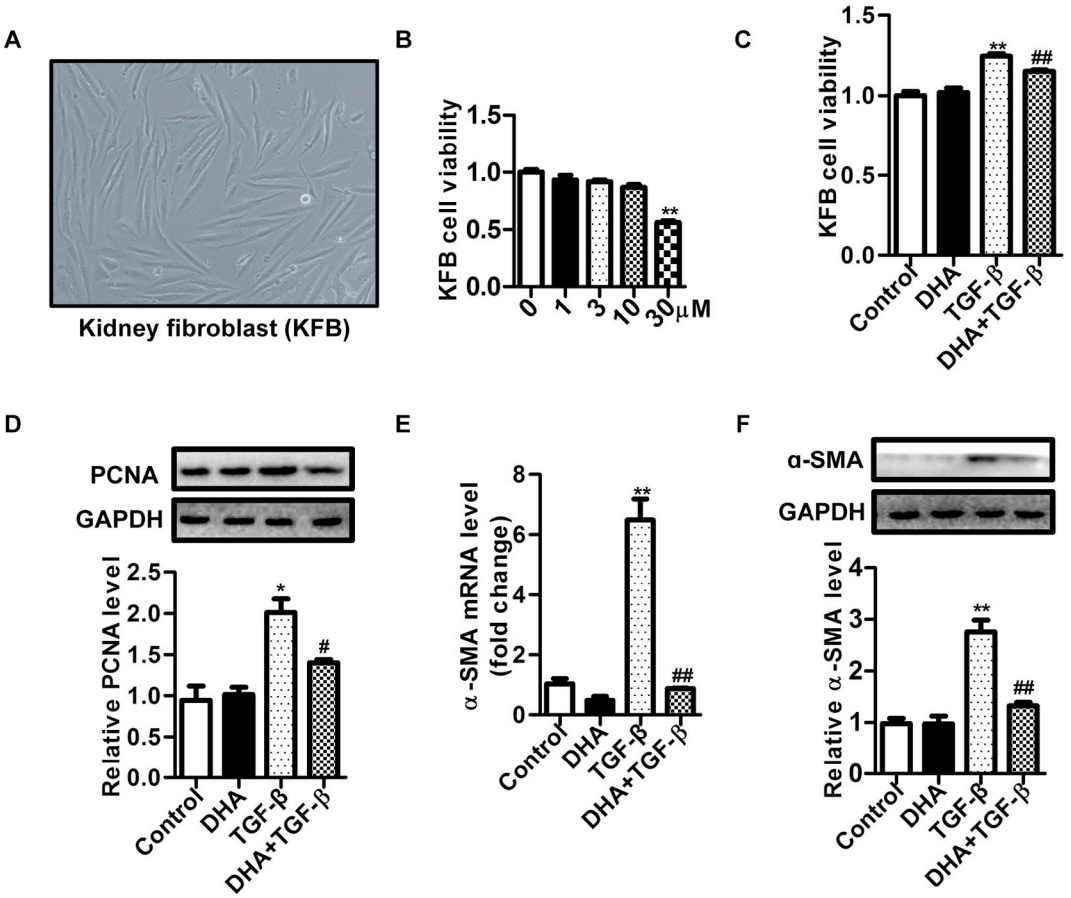

Fig. 5. Effects of DHA on TGF-β1-induced proliferation and differentiation of primary human kidney fibroblasts. (A) Phase contrast microscopic appearance of primary human kidney fibroblasts (Third passages). (B) Viability of primary human kidney fibroblasts, evaluated by the CCK-8 assay under different DHA concentrations. (C) Viability of primary human kidney fibroblasts, evaluated by the CCK-8 assay. (D) Protein expression of PCNA in primary human kidney fibroblasts, determined by western blotting. (E) mRNA levels of α-SMA were quantified by qPCR. (F) Protein expression of α-SMA in primary human kidney fibroblasts, determined by western blotting. The data are presented as the mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 μM group or Control group; $\#P < 0.05$, $\#\#P < 0.01$ versus TGF-β group.

viability of primary human kidney fibroblasts was higher in the TGF-β1 group than in the control but significantly decreased in the DHA + TGF-β1 group compared with that in the TGF-β1 group (Fig. 5C). These data showed that DHA reduced cell viability, which was increased by TGF-β1 treatment but had no significant effect on the viability of fibroblasts without TGF-β1 treatment. Moreover, western blotting analysis indicated that the level of PCNA expression was also significantly lower in the DHA + TGF-β1 group than in the TGF-β1 group (Fig. 5D). In addition, our findings indicated that the mRNA and protein levels of α-SMA were upregulated in TGF-β1-induced primary human kidney fibroblasts but were significantly lower in the DHA + TGF-β1 group (Fig. 5E and F). These data showed that DHA prevented the fibroblasts proliferation as well as fibroblast-to-myofibroblast differentiation induced by TGF-β1 in vitro.

3.6. DHA suppressed the activation of the PI3K/AKT pathway in vivo and in vitro

It has been demonstrated that the PI3K/AKT signaling pathway plays an important role in the progression of renal fibrosis and inflammation [22]. To further investigate the molecular mechanism of the ameliorating effect of DHA on UUO-induced renal fibrosis, we analyzed the effects of DHA on the PI3K/AKT signaling pathway in UUO model and primary human kidney fibroblasts induced by TGF-β1. The protein levels of p-PI3K, PI3K, p-AKT and AKT were measured by western blotting. In vivo, the protein levels of p-PI3K and p-AKT were significantly higher in the UUO group than in the shame group (Fig. 6A), whereas these levels were significantly suppressed in the UUO + DHA group. In vitro, it was shown that the levels of phosphorylation of PI3K and AKT were markedly higher in the TGF-β1 group than in the control group. Whereas phosphorylation of PI3K and AKT was suppressed in the DHA + TGF-β1 group. These results indicate that DHA inhibits activation of the PI3K/AKT signaling pathway in UUO model and TGF-β1-induced primary human kidney fibroblasts.

4. Discussion

The typical feature of renal fibrosis is an excessive deposition of ECM proteins, including collagens and fibronectin, resulting from an imbalance in renal ECM dynamics, wherein the rate of the ECM production exceeds that of its degradation [23]. One of the important results obtained in this study was that the DHA treatment markedly inhibited the upregulation of collagen I, collagen III, and fibronectin, caused by UUO. As a result, we believed that the effects of DHA on renal fibrosis seems directly related to the reduction of ECM components synthesis. Myofibroblasts are a specific cell population thought to be a source of inflammatory cytokines and extracellular matrix in various disease conditions. However, the origin of myofibroblasts in renal fibrosis remains unclear. Using genetic mouse models and a fate-mapping strategy, a previous study found that the origin of myofibroblasts in kidney fibrosis is quite diverse; half of the myofibroblasts accumulated via proliferation and the rest originated from differentiation. Local

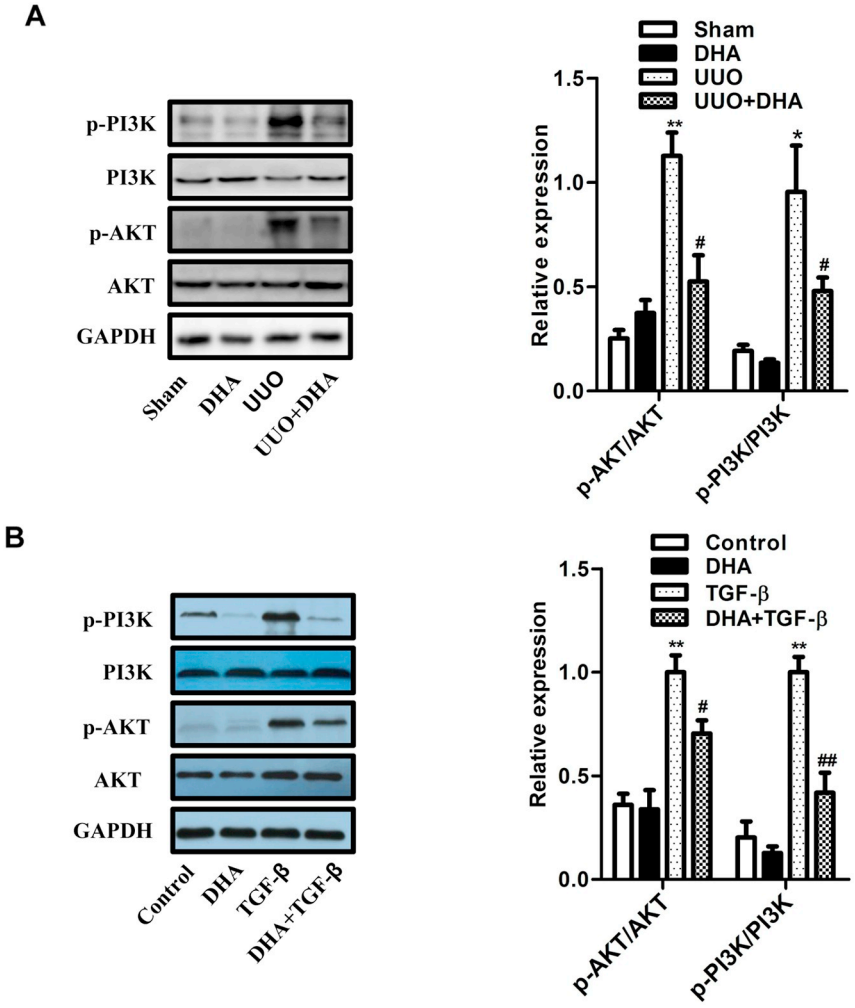

Fig. 6. DHA suppresses the activation of the PI3K/AKT pathway in vivo *and* in vitro.
(A) Left, protein expression levels of p-PI3K, PI3K, p-AKT, and AKT in kidney of mice, determined by western blotting. Right, relative protein expression levels. (B) Left, protein expression levels of p-PI3K, PI3K, p-AKT, and AKT in primary human kidney fibroblasts, determined by western blotting. Right, relative protein expression levels. The data are presented as the mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham group or Control group; $\#P < 0.05$, $\#\#P < 0.01$ versus UUO group or TGF-β group.

fibroblast expansion contributes to about 50% to the myofibroblast pool, while about 35% are recruited from the bone marrow. Endothelial to mesenchymal transition (EndMT) contributes to about 10% and epithelial to mesenchymal transition (EMT) is responsible for about 5% of interstitial myofibroblasts [24]. Obviously, aberrant proliferation of renal fibroblasts and their differentiation to myofibroblasts are hallmarks of progressive renal fibrosis, which is characterized by an excessive ECM build-up leading to the loss of tissue compliance [3,25]. Therefore, inhibition of fibroblast proliferation and differentiation can retard the progression of renal fibrosis. The results of the current study showed that the expression of PCNA in S100A4 positive fibroblast in the kidneys tissue and also the expression of α-SMA increased in UUO mice kidney, while treatment with DHA significantly reduced the expression of PCNA and α-SMA expression in UUO mice. Therefore, one possible mechanism of antifibrotic effects of DHA is through inhibition of fibroblast proliferation and differentiation in renal fibrosis.

Activation of the TGF-β pathway is considered a key event in the development of renal fibrosis. TGF-β1, an attractant for fibroblasts, stimulates these cells to synthesize fibrogenic cytokines and is the most potent profibrotic factor, mediating fibroblast proliferation and activation [26,27]. In our study, when primary human kidney fibroblasts were treated with TGF-β1 (10 ng/mL) for 24 h, cells exhibited a high viability determined by CCK8, higher expression of PCNA and cells differentiated toward a myofibroblast phenotype, characterized by α-SMA expression. While cells pretreatment with DHA was able to inhibit the TGF-β1-induced fibroblast proliferation, as well as their differentiation into myofibroblasts, thus, our findings support that DHA may relieve renal fibrosis through regulation of fibroblast proliferation and differentiation.

The PI3K/AKT signaling pathway plays a central role in cell growth and survival. Researches showed that PI3K/AKT signaling pathway play vital role in the process of renal cell carcinoma cells epithelial-mesenchymal transition (EMT) [28] and PI3K/AKT/mTOR signaling pathway participated in the autophagy of human gingival fibroblasts [29]. Another study on fibrosis signal transduction has shown that the PI3K/AKT signaling pathway can promote the proliferation of human lung fibroblasts and their differentiation into myofibroblasts [13]. Our results showed that the phosphorylation levels of PI3K and AKT in the kidney tissue induced by UUO and in primary human kidney fibroblasts induced by TGF-β1 were significantly downregulated by the treatment with DHA, indicating that the mechanism of the DHA inhibitory effects on renal fibrosis and the primary human kidney fibroblast proliferation and differentiation might involve the inhibition of the PI3K/AKT signaling pathway.

The results of this study demonstrate that DHA, the major active metabolite of artemisinin, is a safe and effective drug, which shows an

attenuating effect on UUO-induced renal fibrosis. In this study, DHA showed an antifibrotic effect and improved the histopathological abnormalities in the UUO model. Furthermore, our results showed that the antifibrotic effect of DHA might be due to the inhibition of fibroblast proliferation and differentiation in vivo and in vitro. Finally, we measured the protein levels of PI3K, p-PI3K, AKT, and p-AKT and found that DHA suppressed the activation of the PI3K/AKT pathway in kidney tissue induced by UUO and in primary human kidney fibroblasts induced by TGF-β1. Thus, our findings showed that DHA might relieve renal fibrosis by mitigating fibroblast proliferation and differentiation via the PI3K/AKT pathway, which can be a new potential therapy of treatment for kidney fibrosis.

5. Conclusion

In conclusion, the present study indicated that DHA could protect against UUO-induced renal fibrosis and this effect might be associated with the reduction of fibroblast proliferation and differentiation via the PI3K/AKT pathway. Thus, DHA may potentially become a preventive or therapeutic drug for the treatment of renal fibrosis.

Author contributions

Conceived and designed the experiments: Xiang Chen, Bo Zhang, Peihua Liu, Yan Zhou. Performed the experiments: Bo Zhang, Peihua Liu, Yan Zhou , Guoyu Dai, Weiping Xia, Yongchao Du. Analyzed the data: Zhi Chen, Yao He. Contributed reagents/materials/analysis tools: Miao Mo, Yuhang Liu. Wrote the paper: Bo Zhang, Peihua Liu, Yan Zhou, Xiang Chen.

Bo Zhang, Peihua Liu, Yan Zhou contributed equally to this work.

Disclosure statement

None of the authors have any conflicts of interest disclose.

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References

[1] M. Liu, T. Liu, P. Shang, Y. Zhang, L. Liu, S. Sun, Acetyl-11-keto-beta-boswellic acid ameliorates renal interstitial fibrosis via Klotho/TGF-beta/Smad signalling pathway, J. Cell. Mol. Med. 22 (10) (2018) 4997–5007.

[2] Dou F, Liu Y, Liu L, Wang J, Sun T, Mu F, Guo Q, Guo C, Jia N, Liu W et al: Aloe-emodin ameliorates renal fibrosis via inhibiting PI3K/Akt/mTOR signaling pathway in vivo and in vitro. Rejuvenation Res. 2018 Oct 24. doi: https://doi.org/10.1089/rej.2018.2104. (Epub ahead of print).

[3] M. Zeisberg, F. Strutz, G.A. Muller, Role of fibroblast activation in inducing interstitial fibrosis, J Nephrol 13 (Suppl. 3) (2000) S111–S120.

[4] W.R. Coward, G. Saini, G. Jenkins, The pathogenesis of idiopathic pulmonary fibrosis, Ther. Adv. Respir. Dis. 4 (6) (2010) 367–388.

[5] K.M. Kim, K.W. Chung, H.O. Jeong, B. Lee, D.H. Kim, J.W. Park, S.M. Kim, B.P. Yu, H.Y. Chung, MMP2-A2M interaction increases ECM accumulation in aged rat kidney and its modulation by calorie restriction, Oncotarget 9 (5) (2018) 5588–5599.

[6] L. Ma, H. Li, S. Zhang, X. Xiong, K. Chen, P. Jiang, K. Jiang, G. Deng, Emodin ameliorates renal fibrosis in rats via TGF-beta1/Smad signaling pathway and function study of Smurf 2, Int. Urol. Nephrol. 50 (2) (2018) 373–382.

[7] G. Qin, C. Zhao, L. Zhang, H. Liu, Y. Quan, L. Chai, S. Wu, X. Wang, T. Chen, Dihydroartemisinin induces apoptosis preferentially via a bim-mediated intrinsic pathway in hepatocarcinoma cells, Apoptosis 20 (8) (2015) 1072–1086.

[8] C. Jiang, S. Li, Y. Li, Y. Bai, Anticancer effects of dihydroartemisinin on human esophageal cancer cells in vivo, Anal Cell Pathol (Amst) 2018 (2018) 8759745.

[9] X.G. Zhang, G.X. Li, S.S. Zhao, F.L. Xu, Y.H. Wang, W. Wang, A review of dihydroartemisinin as another gift from traditional Chinese medicine not only for malaria control but also for schistosomiasis control, Parasitol. Res. 113 (5) (2014) 1769–1773.

[10] Z. Zhang, M. Guo, S. Zhao, J. Shao, S. Zheng, ROS-JNK1/2-dependent activation of autophagy is required for the induction of anti-inflammatory effect of dihydroartemisinin in liver fibrosis, Free Radic. Biol. Med. 101 (2016) 272–283.

[11] D. Yang, W. Yuan, C. Lv, N. Li, T. Liu, L. Wang, Y. Sun, X. Qiu, Q. Fu, Dihydroartemisinin suppresses inflammation and fibrosis in bleomycin-induced pulmonary fibrosis in rats, International Journal of Clinical & Experimental Pathology 8 (2) (2015) 1270–1281.

[12] D.X. Yang, J. Qiu, H.H. Zhou, Y. Yu, D.L. Zhou, Y. Xu, M.Z. Zhu, X.P. Ge, J.M. Li, C.J. Lv, H.Q. Zhang, W.D. Yuan, Dihydroartemisinin alleviates oxidative stress in bleomycin-induced pulmonary fibrosis, Life Sci. 205 (2018) 176–183 S0024320518302790.

[13] E. Conte, M. Fruciano, E. Fagone, E. Gili, F. Caraci, M. Iemmolo, N. Crimi, C. Vancheri, Inhibition of PI3K prevents the proliferation and differentiation of human lung fibroblasts into myofibroblasts: the role of class I P110 isoforms, PLoS One 6 (10) (2011) e24663.

[14] C. Huang, M.Z. Lin, D. Cheng, F. Braet, C.A. Pollock, X.M. Chen, KCa3.1 mediates dysfunction of tubular autophagy in diabetic kidneys via PI3k/Akt/mTOR signaling pathways, Sci. Rep. 6 (2016) 23884.

[15] X. Lin, F. Bai, J. Nie, S. Lu, C. Lu, X. Zhu, J. Wei, Z. Lu, Q. Huang, Didymin alleviates hepatic fibrosis through inhibiting ERK and PI3K/Akt pathways via regulation of Raf kinase inhibitor protein, Cell. Physiol. Biochem. 40 (6) (2016) 1422–1432.

[16] R.L. Chevalier, M.S. Forbes, B.A. Thornhill, Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy, Kidney Int. 75 (11) (2009) 1145–1152.

[17] W. Mckleroy, T.H. Lee, K. Atabai, Always cleave up your mess: targeting collagen degradation to treat tissue fibrosis, American Journal of Physiology Lung Cellular & Molecular Physiology 304 (11) (2013) L709–L721.

[18] F. Strutz, H. Okada, C.W. Lo, T. Danoff, R.L. Carone, J.E. Tomaszewski, E.G. Neilson, Identification and characterization of a fibroblast marker: FSP1, J. Cell Biol. 130 (2) (1995) 393–405.

[19] C.P. Chiang, M.J. Lang, B.Y. Liu, J.T. Wang, J.S. Leu, L.J. Hahn, M.Y. Kuo, Expression of proliferating cell nuclear antigen (PCNA) in oral submucous fibrosis, oral epithelial hyperkeratosis and oral epithelial dysplasia in Taiwan, Oral Oncol. 36 (4) (2000) 353–359.

[20] M. Srinivasan, S.D. Jewell, Quantitative estimation of PCNA, c-myc, EGFR and TGF-alpha in oral submucous fibrosis-an immunohistochemical study, Oral Oncol. 37 (5) (2001) 461–467.

[21] Meng XM, Nikolic-Paterson DJ, Lan HY: TGF-beta: the master regulator of fibrosis. Nat Rev Nephrol 2016, 12(6):325–338.

[22] R. Du, L. Xia, X. Ning, L. Liu, W. Sun, C. Huang, H. Wang, S. Sun, Hypoxia-induced Bmi1 promotes renal tubular epithelial cell-mesenchymal transition and renal fibrosis via PI3K/Akt signal, Mol. Biol. Cell 25 (17) (2014) 2650–2659.

[23] A.A. Eddy, Molecular basis of renal fibrosis, Pediatr. Nephrol. 15 (3–4) (2000) 290–301.

[24] V.S. LeBleu, G. Taduri, J. O'Connell, Y. Teng, V.G. Cooke, C. Woda, H. Sugimoto, R. Kalluri, Origin and function of myofibroblasts in kidney fibrosis, Nature Med 19 (8) (2013) 1047–1053.

[25] F. Strutz, M. Zeisberg, Renal fibroblasts and myofibroblasts in chronic kidney disease, J. Am. Soc. Nephrol. 17 (11) (2006) 2992–2998.

[26] W. Yang, X. Li, S. Qi, K. Zhou, S. Qing, Y. Zhang, M.Q. Gao, lncRNA H19 is involved in TGF-beta1-induced epithelial to mesenchymal transition in bovine epithelial cells through PI3K/AKT signaling pathway, PeerJ 5 (2017) e3950.

[27] W.A. Border, N.A. Noble, TGF-beta in kidney fibrosis: a target for gene therapy, Kidney Int. 51 (5) (1997) 1388–1396.

[28] G.W. Zhang, X. Tian, Y. Li, Z.Q. Wang, X.D. Li, C.Y. Zhu, Down-regulation of ETS2 inhibits the invasion and metastasis of renal cell carcinoma cells by inducing EMT, via, the PI3K/Akt signaling pathway, Biomed. Pharmacother. 104 (2018) 119–126.

[29] J. Liu, X. Wang, M. Zheng, Q. Luan, Lipopolysaccharide from Porphyromonas gingivalis promotes autophagy of human gingival fibroblasts through the PI3K/Akt/mTOR signaling pathway, Life Sci. 211 (2018) 133–139.