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Original Research Article

Therapeutic effect of quinolone oxizime on fibrotic kidney diseases

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Abstract

Purpose: *To investigate the anti-fibrotic effect and mechanism of action of quinolone oxizime in vitro and in vivo.*
Methods: *Proliferation of renal fibroblasts was determined using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while laser confocal fluorescence microscope was used for immuno-cytochemical studies. Total TGF β1 was determined by enzyme linked immunosorbent assay (ELISA).*
Results: *Quinolone oxizime decreased the proliferation of renal fibroblasts in a dose-dependent manner ($p < 0.05$) in vitro. Proliferation of renal fibroblasts was 39, 63, 82, 95, 97 and 99 % on treatment with quinolone oxizime at doses of 10, 8, 6, 4, 2 and 1 µM, respectively, after 48 h. The expression of TGF β1 in the peripheral blood lymphocytes was reduced significantly by quinolone oxizime treatment. In the animal model of renal fibrosis, quinolone oxizime treatment decreased development of lesions, prevented tubular dilation and expansion of interstitium. After 30 days of quinolone oxizime treatment, tubulo-interstitial lesions were completely absent in rats in the 5 mg/kg treatment group. Moreover, quinolone oxizime treatment for 30 days inhibited accumulation of extracellular matrix and prevented renal injury in rats.*
Conclusion: *These results show that quinolone oxizime exhibits anti-fibrotic effects through targeting the expression of TGF β1. Therefore, quinolone can potentially be used for the treatment of fibrotic kidney diseases but further studies are required to ascertain this.*

Keywords: *Quinolone oxizime, Anti-fibrosis, Tubulo-interstitium, Interstitium, Fibroblasts*

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INTRODUCTION

Kidney fibrosis is caused by a complex pathological condition involving processes like accumulation of inflammatory cells, aggregation of collagen and dilation of renal tubules [1]. The progression of kidney fibrosis ultimately leads to the development of end-stage renal disease [1]. Studies have revealed that up-regulation of extracellular matrix (ECM) generation in renal tubules causes tissue scarring and formation of lesions [2, 3]. Production of ECM and onset of fibrosis in the kidneys results in renal fibroblasts [4]. Thus, up-regulation of kidney fibroblast proliferation is regarded as the major factor responsible for pathogenesis of fibrosis [4].

Renal injury leads to increased expression of transforming growth factor (TGF)-β1 [5]. Up-regulation of TGF-β is an important event responsible for the development of kidney fibrosis [6]. Mechanistic studies have shown that the expression of TGF-β activates interstitial fibroblasts which then generates various components of matrix and ultimately results in renal fibrosis. These processes collectively cause tubule-interstitial fibrosis, and subsequently lead to end-stage renal disease [7]. In animal experimental models of kidney disease, these processes are inhibited by chemotherapeutic agents through targeting the renin-angiotensin-aldosterone system [8].

Studies on human beings have also demonstrated that immunosuppressive drugs prevent the progression of kidney diseases [9,10]. These treatment strategies enhance reno-protection but are ineffective against the development of kidney fibrosis. Therefore, the development of an effective treatment strategy for kidney fibrosis is of immense importance.

In the present study, the effect of quinolone oxizime on renal fibrosis was studied *in vitro* in fibroblasts and *in vivo* in a rat model. In rats, the effect of quinolone oxizime on renal fibrosis was investigated after unilateral ureteral obstruction

EXPERIMENTAL

Animals

Male Sprague Dawley rats (250 - 280 g in weight) were purchased from the Animal Centre of Sun Yat-Sen University (Guangzhou, China). All the animals were housed individually in cages and provided with free access to laboratory chow and tap water. The animals were housed under conditions of 12-h/12-h light/dark cycle at 24 ^{o}C and 60 % humidity. The experimental procedures involving animals were performed in accordance with the guidelines of the National Institute of Health on the care and use of laboratory animals. The study has been approved by the ethics committee of Zhongnan Hospital of Wuhan University (approval no. TX1376656). The experimental procedures involving animals were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China [11].

Isolation of kidney fibroblasts and culture

The rats were sacrificed under sodium sorbitol anaesthesia, and the kidneys were surgically excised. The kidneys were sliced into thin (1 mm^3) sections and the sections put into 25 cm^2 containers in Dulbecco's modified Eagle's medium (DMEM; Gibco Corp., Carlsbad, CA, USA). The medium contained fetal calf serum (20 %; FCS; Cultilab, Campinas, Brazil), ampicillin (100 mg/ml) and streptomycin (100 mg/mL).

The culturing was performed under humidified atmosphere of 5 % CO_2 at 37 ˚C. After induction of outgrowths in the explants, undesired tissues were discarded, the cells were harvested at confluence. Immunocytochemistry was used for morphological characterization of the fibroblasts using antibodies against vimentin, desmin and keratin (Sigma Aldrich, St. Louis, MO, USA).

Immuno-cytochemical staining

After attaining confluence, the cells were grown on glass coverslips, incubated for 24 h and then fixed for 30 min using paraformaldehyde (4 %). The cells were then subjected to permeabilization for 20 min at room temperature with Triton X-100 (1 %), washed and blocked for 25 min at room temperature with goat serum (10 %). Following blocking, the cells were treated with antibodies against vimentin, desmin and keratin (Sigma Aldrich, St. Louis, MO, USA) overnight at room temperature.

Thereafter, the cells were washed twice for 15 min with PBS, then incubated for 1 h with horseradish-conjugated secondary antibody. After staining with 3, 3'-diaminobenzidine and hematoxylin for 15 min, the slides were mounted with coverslips and visualized under Zeiss LSM 710 laser confocal fluorescence microscope (Zeiss, Oberkochen, Germany).

Assay of fibroblast proliferation

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay was used for analysis of proliferation in fibroblasts after quinolone oxizime treatment. The fibroblasts were grown separately in medium containing 1, 2, 4, 6, 8 or 10 µM quinolone oxizime for 48 h. After incubation, 5 mg/mL MTT (10 µL) solution in phosphate-buffered saline (PBS) was added to each well of the plate, and incubation was continued for 4 h.

The medium was then discarded, and 150 µL of DMSO was added to each well of the plate and incubated for 10 min. The absorbance of each well was measured at 570 nm using an EL800 Universal Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Enzyme-linked immunosorbent assay (ELISA) for total TGF-β1

The peripheral blood cells obtained were distributed at the density of $1x10^5$cells/mL. The cells were treated with 1, 2, 4, 6, 8 and 10 µM quinolone oxizime for 48 h. After incubation, the cell suspension was centrifuged at 12,000 x g for 50 min to remove insoluble material. Commercially available ELISA kit was used for determination of total TGF-β1 in the supernatant in accordance with the instructions of the kit manufacturer.

Establishment of unilateral ureteral obstruction rat model

Unilateral ureteral obstruction rat model was established using the procedure reported earlier [12]. Twenty rats were randomly assigned to four groups of 5 each: normal control, untreated control, and 2 mg/kg and 5 mg/kg quinolone oxizime treatment groups. The rats in untreated control, and 2 mg/kg and 5 mg/kg treatment groups were given sodium sorbitol anaesthesia and incision was made through the centre of abdominal cavity to isolate and ligate the left ureter of each rat.

The abdominal cavity of each rat was stitched and the animals were kept under sterilized conditions. The rats in the 2 mg/kg and 5 mg/kg quinolone oxizime treatment groups were intraperitoneally injected 2 and 5 mg/kg doses or quinolone oxizime, respectively. After 28 days, the rats were sacrificed under sodium sorbitol anaesthesia, and the kidneys were excised.

Collagen deposition in renal tubules

The excised kidneys were subjected to Masson's trichrome staining for determination of collagen deposition in tubules. Blue coloration in tubules indicated the deposition of collagen. The experiments were performed independently in triplicate, and mean values were obtained.

Statistical analysis

The results presented are mean ± standard deviation (SD, n = 3). Differences among groups were determined statistically using one-way analysis of variance (ANOVA), followed by Tukey post hoc comparison. Statistically significant differences were set at $p < 0.05$. All statistical analyses were done with SPSS 19.0 software (IBM Corp., Armonk, NY, USA).

RESULTS

Culture and characteristics of kidney fibroblasts

The kidney fibroblasts were cultured in DMEM medium for 5 days, and after reaching confluence, were examined using phase contrast microscopy. The morphological examination of the cells revealed presence of kidney fibroblast characteristics (Figure 1). The cells stained positive for vimentin and negative for keramin and desmin.

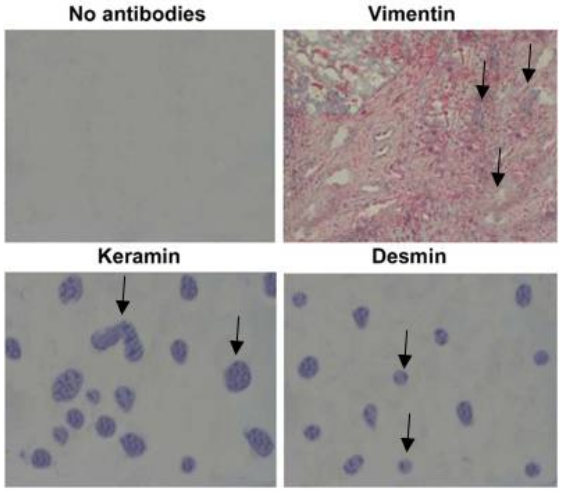

Figure 1: Characteristics of kidney fibroblasts grown from the kidney explants. The cells were cultured for 5 days and subsequently examined for expression of vimentin, keramin and desmin using phase contrast microscopy

Quinolone oxizime inhibited proliferation of kidney fibroblasts

Results from MTT assay showed that quinolone oxizime treatment exhibited inhibitory effect on the proliferation of kidney fibroblasts after 48 h. The kidney fibroblasts were incubated with 1, 2, 4, 6, 8 and 10 µM of quinolone oxizime for 48 h. Significant ($p < 0.05$) decreases were observed in the percentage proliferation of kidney fibroblasts on treatment with 6, 8 and 10 µM doses of quinolone oxizime, when compared to the control fibroblasts (Figure 2). The percentages of fibroblast proliferation were 99, 97, 95, 82, 63 and 39, respectively on treatment with 1, 2, 4, 6, 8 and 10 µM quinolone oxizime, relative to 100 % in control cultures (Figure 2).

Quinolone oxizime inhibited TGF-β1 expression in human lymphocytes

The expression of TGF-β1 secreted by blood lymphocytes for conversion of renal fibroblasts to myofibroblasts, was determined after quinolone

oxizime treatment. The lymphocytes were cultured with 1, 2, 4, 6, 8 and 10 µM of quinolone oxizime for 48 h, and RT-PCR assay revealed significant (p < 0.05) reductions in the expressions of TGF-β1 on treatment with 6, 8 and 10 µM quinolone oxizime in lymphocytes after 48 h, when compared to the control cells (Figure 3).

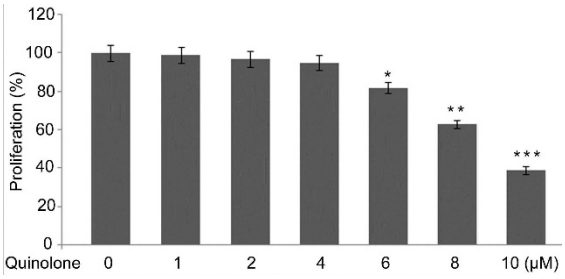

Figure 2: Effect of quinolone oxizime on proliferation of renal fibroblasts. The fibroblasts were treated with various doses of quinolone oxizime for 48 h, and proliferation was measured with MTT assay; *p < 0.05, **p < 0.02 and ***p < 0.02, compared to control fibroblasts

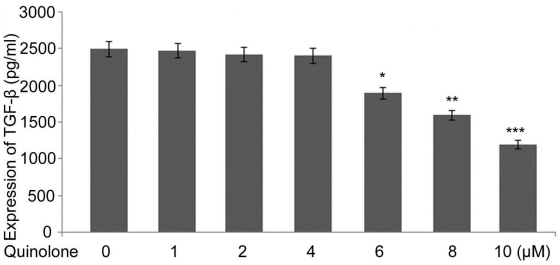

Figure 3: Effect of quinolone oxizime on TGF-β1 expression in lymphocytes. The lymphocytes were cultured with 1, 2, 4, 6, 8 and 10 µM quinolone oxizime and then subjected to RT-PCR for determination of TGF-β1 expression. *p < 0.05 *p < 0.05, **p < 0.02 and ***p < 0.02, compared to control group

Quinolone oxizime prevented collagen deposition in the rat kidney tubules with ureteral obstruction

Effect of quinolone oxizime on the kidneys in rats was studied after 7 days of ureteral obstruction. It was observed that kidneys from untreated rats showed larger deposition of collagen in the tubules than kidneys from treated rats. There was no deposition of collagen in the tubules of rats treated with 5 mg/kg quinolone oxizime (Figure 4).

Quinolone oxizime prevented kidney tubule damage in rats

Kidneys from untreated rats showed large lesions and tubular dilation, when compared to the normal control group. However, treatment with 5 mg/kg doses of quinolone oxizime prevented development of lesions and tubular dilation in the rats (Figure 5). In the kidneys of rats treated with 2 mg/kg quinolone oxizime, some lesion formation and tubular dilation were observed.

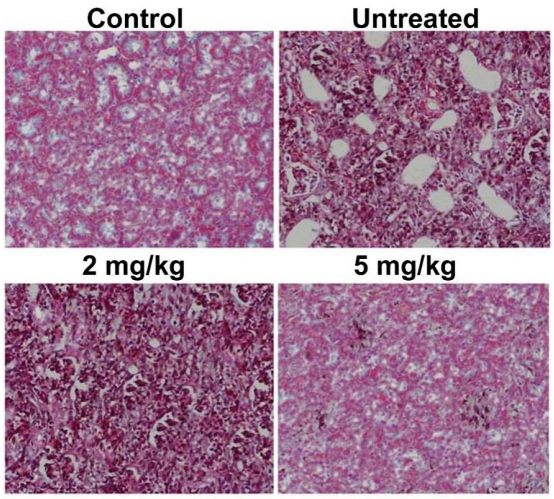

Figure 4: Effect of quinolone oxizime on collagen deposition in the kidney tubules of rats. The collagen deposition in the rat kidney tubules was determined using Masson trichrome staining

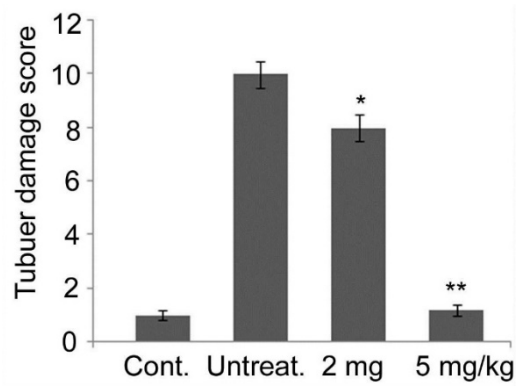

Figure 5: Effect of quinolone oxizime on kidney tubular damage. The rats were treated with 2 and 5 mg/kg doses of quinolone oxizime before examination of tubular damage; *p < 0.05 and **p < 0.02, compared to control animals

DISCUSSION

Renal fibrosis, the advanced stage of tubulo-interstitial lesions is one of the main causes of kidney failure and death [13]. In the present study, effect of quinolone oxizime on renal fibrosis was studied *in vitro* on fibroblasts and *in vivo* in the rat model. It has been reported that the pathogenesis of renal fibrosis is mediated by fibroblasts. Thus, inhibition of their proliferation can be of vital importance for prevention of kidney damage [14]. The present study investigated the effect of quinolone oxizime on

proliferation of kidney fibroblasts cultured from explants using MTT assay. It was found that quinolone oxizime treatment produced dose-dependent inhibitory effect on fibroblast proliferation. There was marked decrease in the proliferation of fibroblasts on treatment with quinolone oxizime for 48 h, when compared to the control cells.

It has been reported that development of kidney interstitial fibrosis is associated with the expression of TGF-β1 (pro-fibrogenic cytokine) [7]. Studies have revealed that TGF-β1/Smad activation plays an important role in the formation of fibres in kidney tubules, and inhibition of Smad antagonist leads to the development of renal interstitial fibrosis [15,16]. To understand the mechanism underlying the inhibition of proliferation by quinolone oxizime treatment, TGF-β1 expression in fibroblasts was determined. The results showed that quinolone oxizime treatment caused reduction in the expression of TGF-β1 in kidney fibroblasts. The maximum reduction of TGF-β1 expression was produced by 30 µM after 48. The myofibroblasts, on activation start to express TGF-β1 which then induces development of kidney interstitial fibrosis. The renal tubular epithelial cells get transformed into myofibroblasts which subsequently generate ECM and develop renal fibrosis [17-19].

In the current study, quinolone oxizime treatment prevented deposition of collagen which forms ECM in the renal tubules in the rats. Quinolone oxizime treatment of the rats also prevented dilation of renal tubules and formation of lesions in the kidneys. These findings suggest that quinolone oxizime exhibits anti-fibrotic effect *in vivo* in the rat model. Kidney fibrosis is caused by the aggregation of collagen, increased level of interstitial cells, and the deformation and dilation of renal tubules [20]. In the present study, after 7-days of UUO surgery showed accumulation of inflammatory cells, kidney tissues of untreated rats had enhanced fibroblast proliferation and deformation of renal tubules. However, quinolone oxizime treatment prevented accumulation of inflammatory cells, inhibited fibroblast proliferation and prevented deformation of renal tubules.

CONCLUSION

The findings of this study demonstrate that quinolone oxizime inhibits proliferation of kidney fibroblasts via the targeting of expression of TGF-β1. In addition, it prevents development of kidney fibrosis in rats by inhibiting transformation of renal epithelial tubular cells into myofibroblasts. Therefore, quinolone oxizime has the potential for use as an anti-fibrotic agent in the management of kidney fibrosis.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qifa Ye designed the study. Sheng Zhang and Qifa Ye together performed the experimental work, literature survey, compiled the data and wrote the paper.

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