



Zinc Deficiency Aggravation of ROS and Inflammatory Injury Leading to Renal Fibrosis in Mice

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Abstract

Zinc (Zn) is a trace element with a variety of anti-inflammatory and antioxidant effects. Zn deficiency is related to tissue fibrosis. The present study was designed to investigate the effect of Zn on renal fibrosis. Mouse models were successfully established by feeding mice diets with different concentrations of Zn. Zn deficiency induced a decrease in Zn levels in kidney tissue. The results also revealed renal vasodilation, hyperemia, and inflammatory cell infiltration, and the levels of creatinine and urea nitrogen were increased. Furthermore, the TUNEL results showed a large degree of renal cell necrosis caused by Zn deficiency. Meanwhile, the corresponding antioxidant and anti-inflammatory regulators (MT-1, MT-2, Nrf2, and TGF- β 1) were detected by RT-PCR, showing that the expression of MT-1, MT-2, and Nrf2 decreased but that TGF- β 1 expression increased. The results of Sirius red staining proved that the expression of collagen was increased by Zn deficiency. The immunohistochemical experiments found that the expression of α -smooth muscle actin (α -SMA) increased. ELISA showed that the expression of Collagen I, III, and IV; fibronectin (FN); and inflammatory factors (TNF- α and IL-1 β) were remarkably increased. The expression of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, and TIMP-1, which are extracellular matrix-regulating molecules, was detected by RT-PCR. The results showed that the expression of TIMPs was increased but that the expression of MMPs was decreased. We also obtained consistent results *in vivo*. All the experimental results indicated that Zn deficiency could aggravate fibrosis by increasing inflammation in the kidney.

Keywords Zn deficiency · Renal fibrosis · Oxidative stress · Inflammation · ECM

Introduction

Chronic kidney disease (CKD) is a highly prevalent disease that represents a serious hazard to both human and animal health. It is estimated that even in developed countries, more than 10% of adults suffer from some degree of CKD [1].

Related studies have shown that progressive renal disease regularly results in renal fibrosis and even renal failure [2]. Progression of CKD is usually caused by two factors: the loss of renal cells and their replacement by extracellular matrix (ECM) [3]. Thus, it is important to study the mechanism of renal fibrosis. At the same time, controlling the development of fibrosis according to its mechanism is also of great interest for controlling the development of CKD.

Some studies have shown that reactive oxygen species (ROS) play an important role in the pathogenesis of renal fibrosis [4–7]. ROS are by-products of cells undergoing aerobic respiration. Under normal body conditions, ROS maintain a dynamic balance [8]; however, studies have shown that the production of ROS in renal fibrosis is obviously excessive. Oxidative stress is viewed as an imbalance between the production of ROS and ROS elimination by protective mechanisms and can lead to inflammation [9, 10]. Therefore, the involvement of the inflammatory response in the formation of renal fibrosis also cannot be ignored [11]. As an important regulatory signal involved in the formation of renal fibrosis, TGF- β 1 is secreted by inflammatory cells and activates

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downstream signalling pathways [12]. TGF- β 1 can stimulate fibroblast proliferation, extracellular matrix synthesis (collagen types I, III, and IV and fibronectin), and epithelial-to-mesenchymal transition (EMT) [13]. When the synthesis and degradation of ECM is out of balance, this can increase the synthesis or reduce the degradation of ECM, which leads to renal fibrosis [3, 14]. In addition to TGF- β 1, there is a major group of enzymes that regulate cell-matrix composition called MMPs. MMPs are Zn-dependent endopeptidases known for their ability to cleave one or more ECM constituents [15]. TIMPs are a kind of enzyme that can regulate MMP activity [16]. When the expression of MMPs is decreased and the expression of TIMPs is increased, the synthesis and degradation of ECM can become unbalanced, which can cause fibrosis.

Many studies have shown that trace elements and some other elements can inhibit or promote inflammation and oxidative stress [17–19]. As one of the most significant trace elements, Zn is involved in numerous biochemical pathways in cells, and it is the only metal that is a cofactor in more than 300 enzymes [20, 21]. Obviously, Zn plays an important role in animals. When the level of Zn is deficient, this can lead to disorders of body function, which can lead to several diseases. Increasing evidence suggests that Zn deficiency increases the concentrations of ROS and inflammatory factors. Zn plays a preventive role in free radical formation resulting from injury during inflammatory processes [22, 23]. Since Zn is widely distributed in various organs and maintains relatively high concentrations in the kidneys, a lack of Zn may exacerbate kidney damage [24]. Therefore, we conducted a study on the effect of Zn deficiency on renal fibrosis.

Materials and Methods

Establishment and Design of the Experimental Animal Model

For the experiment, all mice were provided by the Huazhong Agricultural University Laboratory Animal Research Center (Wuhan, China), which were fed and used in accordance with animal management guidelines. Eighty adult-weaned male BALB/c mice (3–4 weeks old, weighing 20–25 g) were divided into three equal groups. One group of mice was fed a diet with normal Zn levels (34 mg/kg). One group was fed a diet with a high Zn level (100 mg/kg), and the remaining group was fed a diet with a deficient Zn level (2 mg/kg). The rat food was provided by the Nantong Trophi Feed Company. All mice were kept and fed in a sunny, temperature-controlled room for 6 weeks. After the mice were killed, their kidney tissues were divided into three groups as follows: the Zn-deficient group (ZnL, 2 mg Zn/kg), Zn-normal group (ZnM, 34 mg Zn/kg),

and Zn-high group (ZnH, 100 mg Zn/kg). The kidney tissue was placed in liquid nitrogen and stored at -80°C .

Histopathological Analysis

Appropriate amounts of kidney tissues were obtained and fixed in 10% formalin for 1 day. Paraffin-embedded samples were obtained, deparaffinized with xylene, and rehydrated with a graded alcohol series prior to staining and analysis. The sections were stained with hematoxylin and eosin.

Sirius Red Staining

Sirius red was used to stain the paraffin sections with a thickness of 4 μm , which were prepared by gradient dehydration, transparentizing, and embedding. Paraffin was removed with toluene-anhydrous alcohol. Then, the tissues were rehydrated by placing them in anhydrous alcohol, 70% alcohol, 50% alcohol, and finally water. The tissue sections were stained with a mixture of 0.1% Sirius red F3BA and 0.25% Fast Green FCF at room temperature for 45 min. Then, they were dehydrated, transparentized, and sealed. The deposition of the fibrous component Collagens (dyed red) in kidney tissues was observed under an optical microscope, and semiquantitative analysis was conducted using a digital image collection and analysis system.

Immunohistochemistry

The kidney tissue block was routinely dewaxed and hydrated, and nonspecific protein binding was blocked after antigen retrieval. The primary antibody, which can specifically bind to α -SMA, was incubated with the tissue at 37°C for 1 h or overnight at 4°C . Then, the tissue was rewarmed at 37°C for 45 min. The secondary antibody with a detectable label was incubated with the tissue at 37°C for 30 min to 1 h. Then, we stained the tissue with DAB and added ddH₂O. The samples were stained with hematoxylin, decolorized, and transparentized. After drying, the neutral resin sealant was observed under a light microscope.

ROS Detection

Fresh tissue samples were washed with PBS, after that weigh 100 mg of tissue samples accurately, and add 1 ml buffer A homogenizing thoroughly with glass homogenizer. Centrifugation was performed at the condition of 4°C , 1000 g for 10 min. After abandoning the precipitate, we took the supernatant. Add 200 μl supernatant of homogenate and 2 μl 013 probe to 96-well plate, and blow them with pipette to make them fully mixed. Incubate it at 37°C in the dark for 30 min. The 96-well plate was placed in a fluoroenzyme labeling instrument, and the excitation wavelength was 488, and the

emission wavelength was 610 nm to detect the fluorescence intensity. Another 50 μl supernatant homogenate was taken and diluted with PBS for about 30 times, 100 μl of which was used for protein quantification. DHE ROS kit was used for testing. The DHE solution was diluted to the appropriate concentration to replace the cell culture solution. The HEK293T cells were incubated at 37 °C for 10–90 min in dark and then washed with fresh solution. Fluorescence microscopy was used for detection.

Measurement of Creatinine and BUN

Creatinine and BUN were the blood evaluation index of renal function [25]. Blood samples from the mice were taken by eye enucleation. After all the mice were fixed, surgical clippings were used to remove their whiskers to prevent blood from causing hemolysis. Use tweezers to remove the eyeballs quickly, and make blood flow from the orbit into the EP tubes. Put them in ice bath for 30 min, and then use the centrifugal to separate the supernatant. Blood routine examination of creatinine and urea nitrogen was performed to compare normal values.

HEK 293T Cell Culture

The HEK293T cell line was cultured in the presence of 10% fetal bovine serum. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml Streptomycin, at 37 °C and 5% CO₂. After the cells are grown in the cell flask, they are inoculated in a six-well plate. Three groups of serum-free culture medium with successively reduced zinc concentration were prepared (0, 10, 25 $\mu\text{M/L}$).

Quantitative Real-Time PCR Analysis

Total RNA was extracted from kidney tissue and HEK293T cells, and the concentration and purity of RNA were determined by ultraviolet spectrophotometry (260/280 ratio). Equal amounts of RNA were then reverse-transcribed to synthesize complementary DNA (cDNA of the sample). Then, we selected and designed primers and used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control gene; reactions were carried out using the SYBR Green I system. Finally, a Roche Light Cycler 96 instrument that was developed in Switzerland was used for the determination of the target gene expression levels (TIMP-1; MMP-1, 3, 7, 9, 12; MT-1, 2; TGF- β 1; IL-1 β ; TNF- α). Each sample was repeated three times. GAPDH was used as an internal reference gene. The relative abundance of mRNA for each gene was calculated according to the $2^{-\Delta\Delta C_t}$ method as the previous study [26].

ELISA

Kidney tissues were weighed, homogenized with phosphate-buffered saline on ice, and centrifuged at 2000 rpm for 40 min at 4 °C and then the supernatants were collected. All the supernatants were stored at -20 °C prior to measurements. HEK293T cells were grown and then incubated in serum-free RPMI. The supernatant was then harvested for ELISA. The inflammatory cytokines (TNF- α , IL-1 β) and the proteins associated with fibrosis (Collagen I, III, IV, and FN) were detected with mouse ELISA kits in accordance with the instructions of the microplate reader to get the OD value under the conditions of 450 nm within 15 mins. Meanwhile, the results are expressed as the mean \pm standard deviation of all the independent experiments.

Data analysis

All statistical analyses were performed using the SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). *T* tests were conducted, and results were represented in the form of mean \pm standard deviation (M \pm SD). Samples with different superscript letters indicate that there is significant difference among ZnL, ZnM, and ZnH. ($p < 0.05$).

Results

In vivo

Effects of Zn Deficiency on Injury and Correlation of Regulators

Blood from mice was collected by enucleation of the eyeball. Meanwhile, renal function examination was performed on three groups of samples, and the results showed that (Fig. 1a) the creatinine and urea nitrogen levels in ZnL were higher than those in ZnM and ZnH. Furthermore, ROS kits were used for detection. The ROS results are shown in Fig. 1b. Compared with that in ZnM, the ROS content in ZnL increased significantly, but the increase in ZnH was not obvious, with no significant change. Inflammatory factors were detected by ELISA. It was obvious that compared with those in ZnM, the inflammatory factors in ZnL increased significantly, while those in ZnH did not.

The kidney sections subjected to H&E staining are shown in Fig. 1d–f. Compared with ZnM, ZnL showed obvious vascular congestion, renal corneal injury with loss of filtration space, and infiltration of many inflammatory cells. There was no significant difference between ZnH and the other groups.

Figure 1 g and h shows that four genes were found with changes in the kidneys of mice. Among the four genes, one

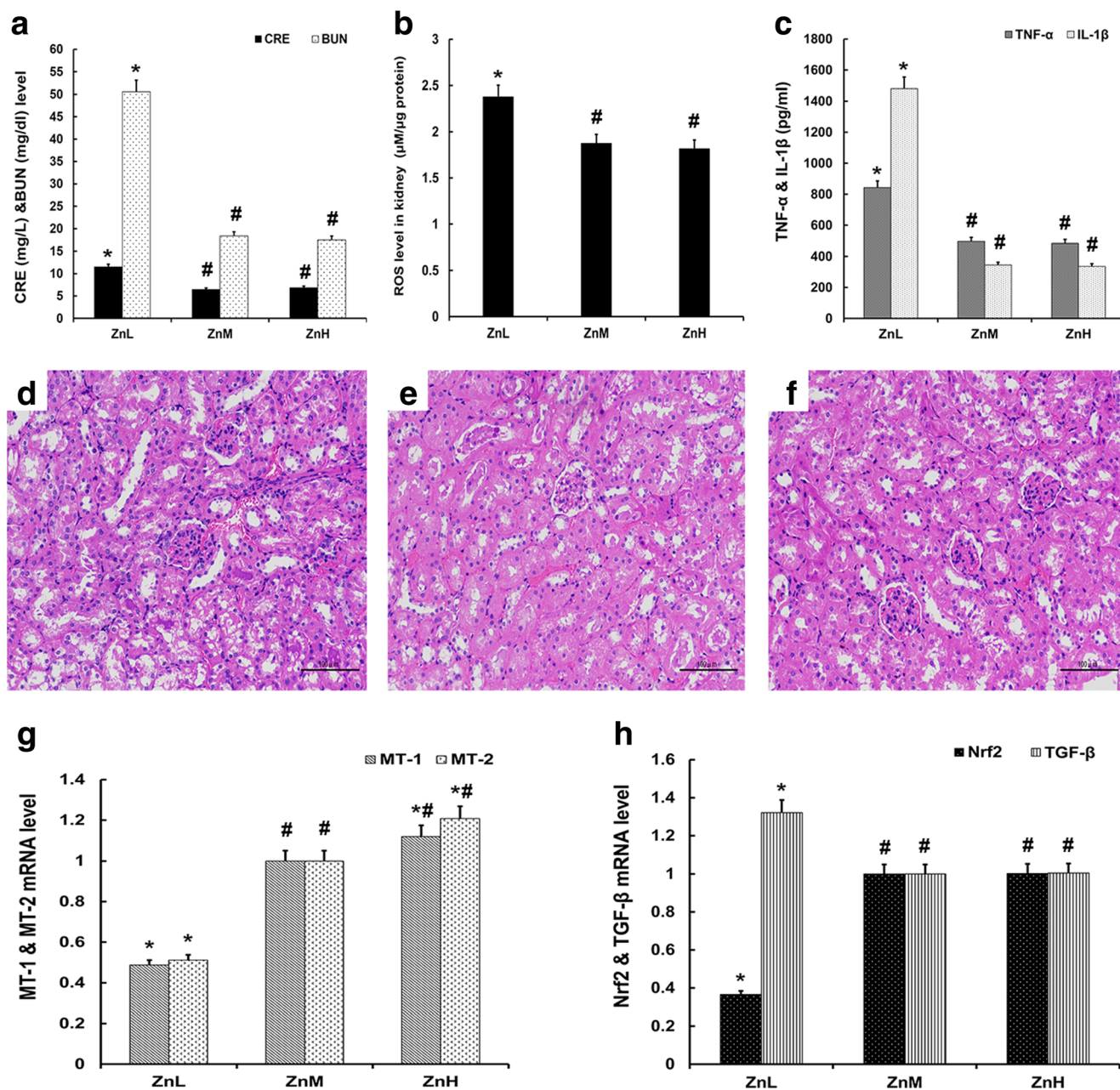


Fig. 1 Analysis of injury and the correlation of regulators in the kidney. Protein expression was detected with ELISA kits. RT-qPCR was used to verify mRNA expression. GAPDH was used as a control. **a** Results showing CRE and BUN content. **b** The results showing reactive oxygen species (ROS) levels. **c** TNF- α and IL-1 β protein content. **d** Results of H&E staining of kidney tissue, ZnL. **e** Results of H&E staining of kidney tissue, ZnM. **f** Results of H&E staining of kidney tissue, ZnH. **g** The

mRNA levels of MT-1 and MT-2. **h** The mRNA levels of Nrf₂ and TGF- β 1. ZnL (Zn-low, 2 mg Zn/kg). ZnM (Zn-normal, 34 mg Zn/kg). ZnH (Zn-high, 100 mg Zn/kg). Asterisk represents a comparison with ZnM. Number sign represents a comparison with ZnL. The values are presented as the mean \pm SD of three independent experiments. $P < 0.05$, significantly different from ZnM

was upregulated, and the other three were downregulated. To further confirm the effects of Zn on these genes, RT-qPCR was carried out. The results showed that when the level of Zn decreased, the mRNA levels of TGF- β 1 were significantly increased ($P < 0.05$) and those of MT-1, MT-2, and Nrf₂ were significantly decreased ($P < 0.05$). In addition, when the Zn content increased, the mRNA levels of the MTs increased

significantly, but the changes in Nrf₂ and TGF- β 1 were not obvious.

Correlation Between Zn Deficiency and Renal Fibrosis

Sirius red staining was performed on to determine the kidney tissue fibrosis index based on collagen to observe renal

Table 1 The primer for qPCR

Target gene	Primer sequence (5'-3')
GAPDH	Forward: 5'- GCACAGTCAAGGCCGAGAAT -3' Reverse: 5'- GCCTTCTCCATGGTGGTGAA -3'
MMP-1	Forward: 5'- ACTTTGTTGCCAATTCCAGG -3' Reverse: 5'- TTTGAGAACACGGGGAAGAC -3'
MMP-3	Forward: 5'- TGTCCCGTTTCCATCTCTCTC -3' Reverse: 5'- TGGTGATGTCTCAGGTTCCAG -3'
MMP-7	Forward: 5'- TTGGCTTCGCAAGGAGA -3' Reverse: 5'- GGGTGGCAGCAAACAGG -3'
MMP-9	Forward: 5'- GGGACCATCATAACATCACA -3' Reverse: 5'- CTCCGCGACACCAAACCT -3'
MMP-12	Forward: 5'- GCTGCTCCCATGAATGA -3' Reverse: 5'- TTGCCAGAGTTGAGTTGTC -3'
TIMP-1	Forward: 5'- CAGAACCGCAGTGAAGAG -3' Reverse: 5'- CAGAACCGCAGTGAAGAG -3'
MT-1	Forward: 5'- GAGTGAGTTGGGACACCTTG -3' Reverse: 5'- CCCTGGGCACATTTGGA -3'
MT-2	Forward: 5'- GACTCTACTAATAAGACCGAATG -3' Reverse: 5'- AGCCCTGGGAGCACTTC -3'
Nrf ₂	Forward: 5'- TGGAGCAGCAGTGGCAGGAC -3' Reverse: 5'- AAGGGTTTCAGCGGTCTGTGTTG -3'
TGF-β1	Forward: 5'- CCCACTGATACGCTGAG -3' Reverse: 5'- GGGCTGATCCCGTTGAT -3'
TNF-α	Forward: 5'- CTCATTCTGCTTGTGGC -3' Reverse: 5'- CACTTGGTGGTTTGTCTACG -3'
IL-1β	Forward: 5'- TTCCATTAGACAACACTGC -3' Reverse: 5'- CTGTAGTGTGTATGTGATC -3'

fibrosis levels by using a digital image acquisition system. The results confirmed that the levels of collagen glomerular deposition in ZnL shown in Fig. 2a were significantly increased compared with those in the other groups (Fig. 2b-c).

Immunohistochemistry showed that α-SMA was expressed in proliferative fibrous tissue in mouse kidney. The results of immunohistochemistry showed that the more severe the Zn deficiency, the more brown-yellow particles that existed, which meant that α-SMA was more highly expressed.

The expression of collagen I, III, and IV and FN were detected by ELISA. The results are shown in Fig. 2g-i. The expression of collagen and fibronectin in ZnL were evidently increased, while the increases in ZnH were not obvious compared with those in ZnM.

Effects of Zn Deficiency on TIMPs and MMPs

The formation of renal fibrosis is related to the regulation of TIMPs and MMPs. The results of the experiments were measured by RT-qPCR. As shown in Fig. 3, compared with that in ZnM, the expression of TIMP-1 in ZnL increased significantly ($P < 0.05$), while expression of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12 decreased significantly ($P < 0.05$).

Moreover, the results showed that TIMP-1 expression decreased with increasing Zn content, while the expression of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12 showed the opposite trend. This also indicated that Zn content can affect the expression of TIMPs and MMPs to some extent.

In Vitro

Effects of Zn Deficiency on Correlation of Regulators, ROS, and Inflammatory Factors

Figure 4 a and b show that expression of four genes were associated with Zn in HEK 293T cells. Among the four genes, one was upregulated, and the other three were downregulated. To further confirm the effects, RT-qPCR was carried out. The results showed that when the level of Zn decreased, the mRNA levels of TGF-β1 were significantly increased ($P < 0.05$) and those of MT-1, MT-2 and Nrf₂ were significantly decreased ($P < 0.05$).

The results of ROS detection in HEK 293T cells are shown as follows (Fig. 3d). Compared with that in ZnH and ZnM, the ROS content in ZnL was significantly increased. To further confirm the effects of Zn deficiency on ROS, the fluorescence intensity in each group was measured. The higher the fluorescence intensity, the higher the content of ROS. The intensity in ZnL was much higher than that in the other two groups. Zn deficiency has been shown to cause an imbalance between ROS production and elimination.

The detection of inflammatory factors in HEK 293T cells was carried out at the gene and protein levels. Figure 4 d illustrates that a total of two inflammatory-related genes were found in HEK 293T cells. Both genes were upregulated. To further confirm the effects of Zn on these genes, RT-qPCR was carried out. The results showed that the mRNA levels of IL-1β and TNF-α were significantly increased ($P < 0.05$) in ZnL. Meanwhile, inflammatory proteins were also detected by ELISA. It was obvious that compared with those in ZnM, the levels of inflammatory proteins in ZnL increased significantly, while those in ZnH did not.

Effects of Zn Deficiency on MMPs and TIMP Expression

Figure 5 shows that six genes were found in HEK 293T cells. Among the six genes, one was upregulated, and the other five were downregulated. To further confirm the effects of Zn on these genes, RT-qPCR was carried out. The results showed that the mRNA levels of TIMP-1 were significantly increased ($P < 0.05$) and those of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12 were significantly decreased ($P < 0.05$) in ZnL HEK 293T cells.

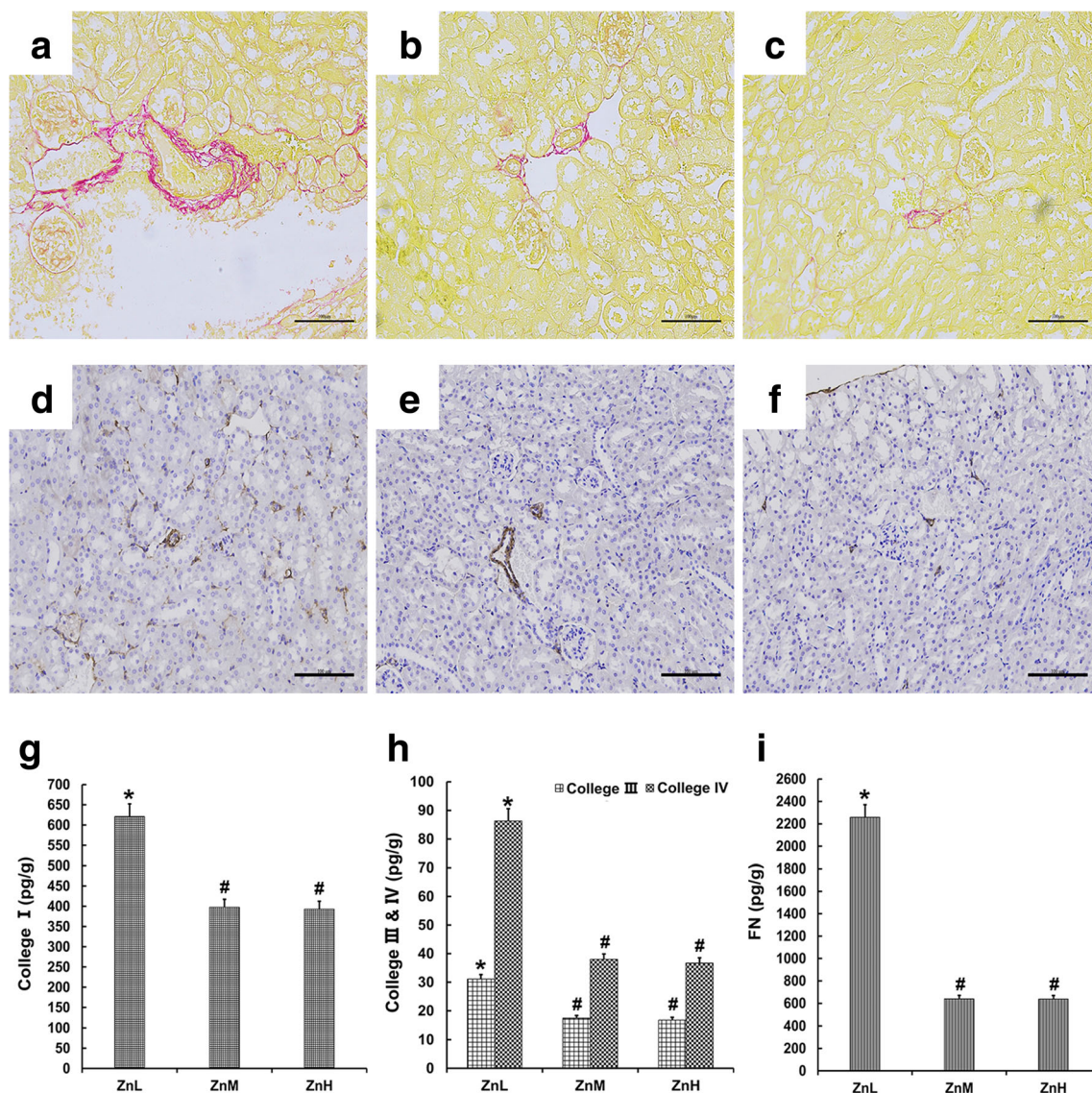


Fig. 2 Fibrosis detection in kidney tissues. The expression of collagen I, III, IV and FN was detected with ELISA kits. **a** The results of Sirius red staining, ZnL. **b** The results of Sirius red staining, ZnM. **c** The results of Sirius red staining, ZnH. Red represents the collagen fibers. **d** The α -SMA immunohistochemistry results, ZnL. **e** The α -SMA immunohistochemistry results, ZnM. **f** The α -SMA immunohistochemistry results, ZnH. Brownish-yellow particles represent α -SMA, and blue represents

the nucleus. **g** The expression of collagen I. **h** The expression of collagen III and IV. **i** The expression of FN. ZnL (Zn-low, 2 mg Zn/kg). ZnM (Zn-normal, 34 mg Zn/kg). ZnH (Zn-high, 100 mg Zn/kg). Asterisk represents a comparison with ZnM. Number sign represents a comparison with ZnL. The values are presented as the mean \pm SD of three independent experiments. $P < 0.05$, significantly different from ZnM.

The Relationship Between Zn Deficiency and Fibrosis

Cellular supernatants were used in cell experiments. The expression of Collagen I, III, and IV and FN was detected by ELISA. The results are shown in Fig. 6. The expression of Collagen I, III, and IV and FN in ZnL were increased. Compared with those in ZnM, the increases in ZnH were not obvious. All the results of the in vitro experiments were consistent with those of the in vivo experiments.

Discussion

Zn is crucial for the functions of numerous proteins that regulate the functions of various other proteins, including metalloproteins. Zn is an essential micronutrient in many cellular processes, so it has important biological significance. In this experiment, a Zn-deficient mouse kidney model was successfully established through a Zn-deficient diet. Studies have shown that when the body is Zn-deficient, it can cause damage to the kidneys [27]. Kidney function was evaluated by

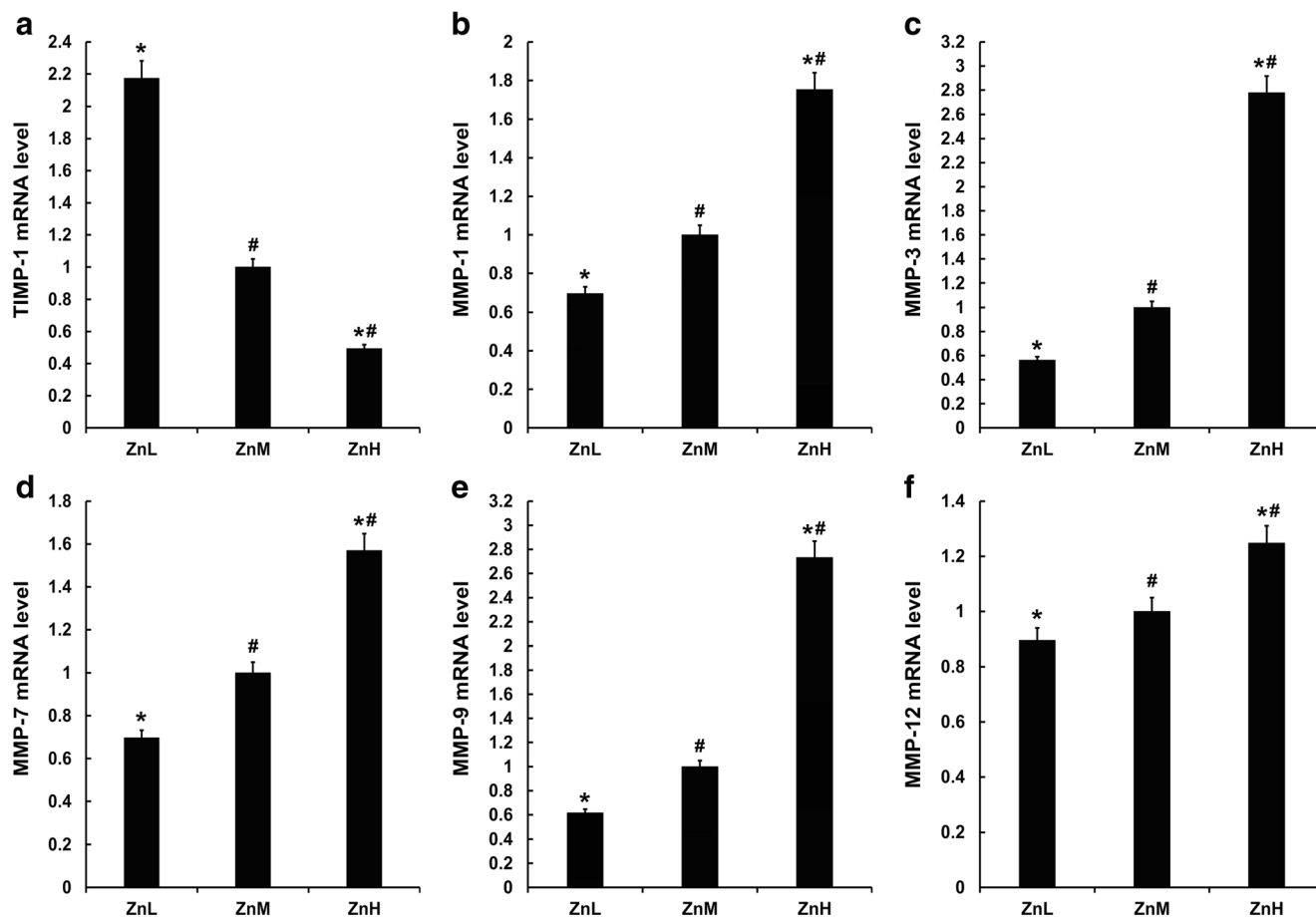


Fig. 3 Expression of TIMPs/MMPs in kidney tissues. RT-qPCR was used to verify the expression of mRNA. GAPDH was used as a control. **a** The mRNA level of TIMP-1. **b** The mRNA level of MMP-1. **c** The mRNA level of MMP-3. **d** The mRNA level of MMP-7. **e** The mRNA level of MMP-9. **f** The mRNA level of MMP-12. ZnL (Zn-low, 2 mg Zn/kg).

ZnM (Zn-normal, 34 mg Zn/kg). ZnH (Zn-high, 100 mg Zn/kg). Asterisk represents a comparison with ZnM. Number sign represents a comparison with ZnL. The values are presented as the mean \pm SD of three independent experiments. $P < 0.05$, significantly different from ZnM

tracking the serum levels of urea and creatinine. These two parameters are usually altered in the presence of substantial damage to kidney nephrons [28]. ZnL showed a significant rise in these two kidney function markers as a consequence of impaired kidney function.

Zn has anti-inflammatory and antioxidant effects [29]; when the body is in a Zn-deficient state, the capacity of anti-inflammatory and antioxidant activities is frequently reduced, which increases the risk of the development of disease. MTs are a class of Zn-binding proteins whose stimulation and expression can significantly reduce ROS levels and regulate inflammation [30, 31]. The transcription factor Nrf₂ is a cellular antioxidant and antioxidant [32, 33]. After activation, Nrf₂ can upregulate its downstream antioxidant factors through nuclear translocation and exert its effects on antioxidant damage. Zn is also a regulator of Nrf₂ [34]. Thus, in this experiment, ROS content was measured in renal tissues and HEK 293T cells by using ROS assay kits. At the same time, the expression levels of MT-1, MT-2, Nrf₂, IL-1 β , and TNF- α were detected by RT-qPCR. The experimental results showed that ROS content

and IL-1 β and TNF- α levels increased significantly in both the ZnL tissue and cell groups. Based on the H&E staining of tissues, it could be easily seen that there was increased inflammatory cell infiltration in the tissue. All of the above results proved that Zn deficiency could cause oxidative damage and inflammation in the kidney. The expression of MT-1, MT-2, and Nrf₂ were decreased in ZnL, indicating that Zn deficiency could reduce the antioxidant capacity of the body.

TGF- β 1 is a major driver of fibrosis via its activation of its downstream effector small mother against decapentaplegic (Smad) signalling [35]. TGF- β 1 exerts its fibrotic activity through stimulation of fibroblast proliferation and extracellular matrix synthesis (collagen I, III, IV and fibronectin) [12]. α -Smooth muscle actin (α SMA) is the most characteristic marker of pathological fibroblasts [36]; therefore, it was detected by immunohistochemistry in this experiment. The expression of TGF- β 1 was obviously increased, which demonstrated the initiation of renal fibrosis. ELISA kits were used to measure collagen (collagen types I, III, IV and fibronectin) expression in tissues and HEK 293T cells. The results in both

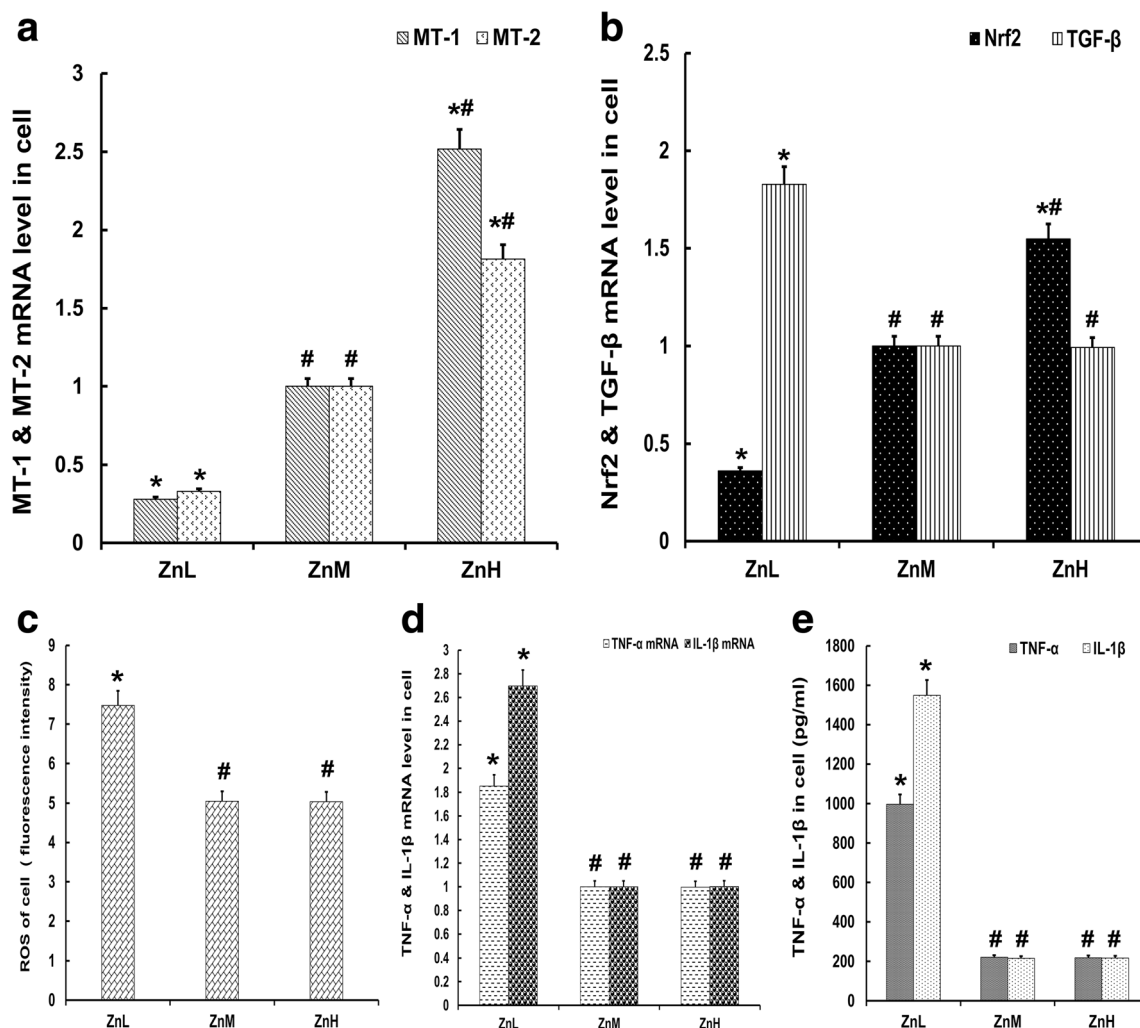


Fig. 4 Analysis of the correlation of regulators, ROS, and inflammatory factors in HEK 239T cells. The qPCR was used to verify the expression of mRNA. GAPDH was used as a control. Protein expression was detected with ELISA kits. **a** The mRNA levels of MT-1 and MT-2. **b** The mRNA levels of Nrf2 and TGF- β 1. **c** The results for ROS levels. **d** The mRNA levels of TNF- α and IL-1 β . **e** TNF- α and IL-1 β protein content. ZnL

(Zn-low, 2 mg Zn/kg). ZnM (Zn-normal, 34 mg Zn/kg). ZnH (Zn-high, 100 mg Zn/kg). Asterisk represents a comparison with ZnM. Number sign represents a comparison with ZnL. The values are presented as the mean \pm SD of three independent experiments. $P < 0.05$, significantly different from ZnM

tissues and cells showed that the expression of collagen in the ZnL group was increased compared with that in the other groups. Immunohistochemistry showed that there were more brown-yellow particles in the ZD group than in the other groups, which indicated that the expression of α -SMA was notably increased. The results of Sirius red staining showed that a large number of collagen fibers were dyed red in ZnL. All of the above results confirm that zinc deficiency indeed caused renal fibrosis.

Renal fibrosis is caused by excessive deposition and modification of extracellular matrix (ECM) in the renal parenchyma in response to injury and inflammation [37]. Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in physiological processes such as tissue repair [38] that can remodel the ECM. TIMPs are inhibitors of MMPs, and TIMPs and MMPs interact with each other to balance ECM

synthesis and degradation [39]. Under normal physiological conditions, their expression and activity are strictly controlled, while under pathological conditions, their expression is often problematic [40]. In this study, the expression of TIMP-1, MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12 in renal tissues and cells was detected by RT-qPCR. The results showed that the expression of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12 in ZnL were decreased compared with those in other groups, while the expression of TIMP-1 in ZnL was increased. Therefore, ECM production and degradation were out of balance, causing renal fibrosis.

Experiments have shown that Zn deficiency in mice can lead to ROS accumulation and an inflammatory response, resulting in renal fibrosis. When chronic kidney disease occurs, the loss of Zn increases, which can accelerate the development of kidney disease in the body. Therefore, appropriate

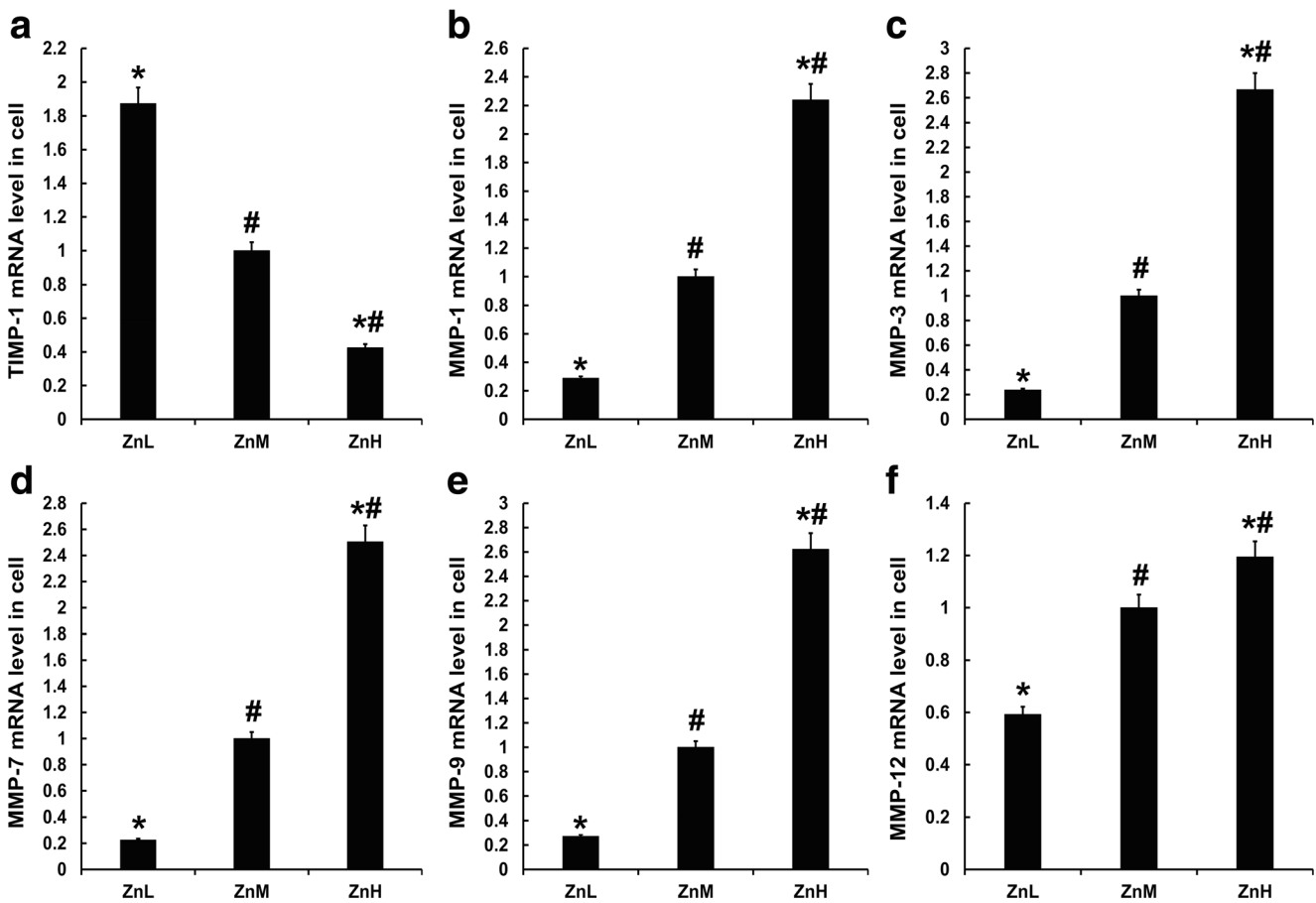


Fig. 5 Expression of TIMPs/MMPs in HEK 239T cells. RT-qPCR was used to verify mRNA expression. GAPDH was used as a control. **a** The mRNA level of TIMP-1. **b** The mRNA level of MMP-1. **c** The mRNA level of MMP-3. **d** The mRNA level of MMP-7. **e** The mRNA level of MMP-9. **f** The mRNA level of MMP-12. ZnL (Zn-low, 2 mg Zn/kg).

ZnM (Zn-normal, 34 mg Zn/kg). ZnH (Zn-high, 100 mg Zn/kg). Asterisk represents a comparison with ZnM. Number sign represents a comparison with ZnL. The values are presented as the mean \pm SD of three independent experiments. $P < 0.05$, significantly different from ZnM

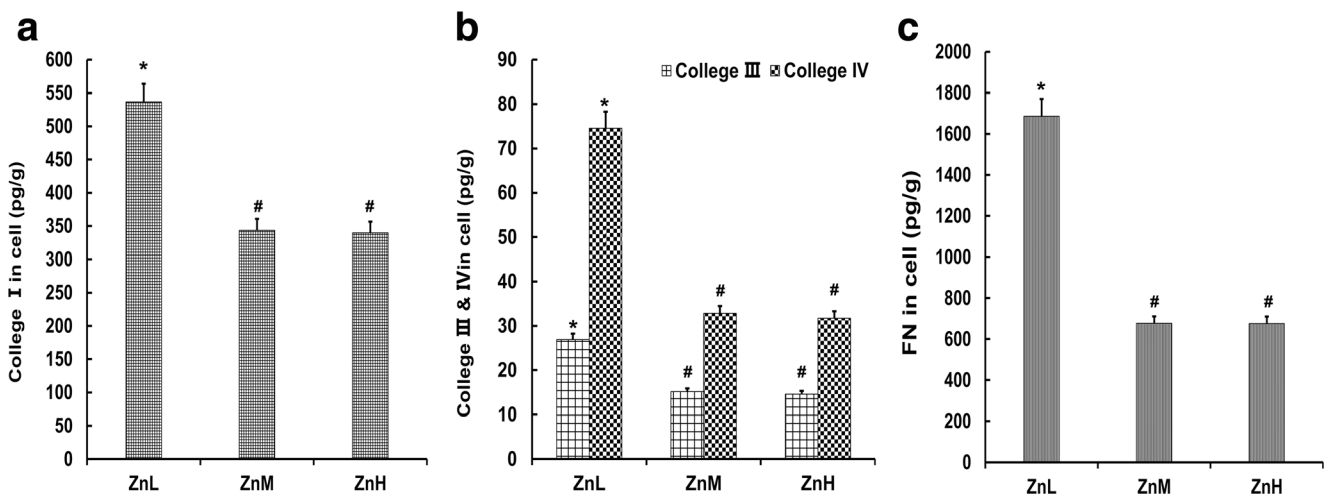


Fig. 6 Analysis of protein content related to fibrosis in HEK 239T cells. The expression of collagen I, III, and IV and FN was detected with ELISA kits. **a** The expression of collagen I. **b** The expression of collagen III and IV. **c** The expression of FN. ZnL (Zn-low, 2 mg Zn/kg). ZnM (Zn-normal,

34 mg Zn/kg). ZnH (Zn-high, 100 mg Zn/kg). Asterisk represents a comparison with ZnM. Number sign represents a comparison with ZnL. The values are presented as the mean \pm SD of three independent experiments. $P < 0.05$, significantly different from ZnM

Zn supplementation for chronic kidney disease may be a new strategy for delaying the development of kidney disease.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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