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The Effect of Overexpression of the Enhancer of Zeste Homolog 1 (EZH1) Gene on Aristolochic Acid-Induced Injury in HK-2 Human Kidney Proximal Tubule Cells *In Vitro*

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Acute kidney injury (AKI) involves the renal tubular epithelium. The enhancer of zeste homolog 1 (EZH1) gene has a role in cell development and differentiation. This study aimed to investigate the effect of overexpression of the EZH1 gene on aristolochic acid-induced injury in HK-2 human kidney proximal tubule epithelial cells *in vitro*.

Material/Methods: The HK-2 cells were cultured and treated with aristolochic acid and the effects of aristolochic acid-injury were evaluated using a cell counting kit-8 (CCK-8) assay. Overexpression of EZH1 used gene plasmid transfection into HK-2 cells. The cell apoptosis rate and levels of intracellular reactive oxygen species (ROS) were measured using flow cytometry. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were performed to determine the expressions of inflammatory cytokines including interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), apoptosis-related genes, and the downstream target genes of NF-κB signaling pathway, including NFKBIA, CXCL8, and cyclin D1.

Results: Aristolochic acid inhibited HK-2 cell viability, induced cell apoptosis, increased the levels of ROS and inflammatory cytokines, including IL-1β, IL-6, TNF-α, and activated the NF-κB pathway. Overexpression the EZH1 gene inhibited HK-2 cell apoptosis, reduced ROS levels, and down-regulated the expressions of IL-1β, IL-6, TNF-α, Bax and Cyt C mRNA and protein, and increased the expressions of Bcl-2 and NFKBIA, CXCL8 and cyclin D1, indicating that overexpression of EZH1 suppressed NF-κB signaling in aristolochic acid-injured HK-2 cells.

Conclusions: Overexpression of EZH1 reduced HK-2 cell injury induced by aristolochic acid *in vitro* by inhibition of NF-κB signaling.

MeSH Keywords: **Acute Kidney Injury • Apoptosis • Aristolochic Acids • EZH1 • NF-kappa B**

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Background

Acute kidney injury (AKI) is characterized by a rapid decline in renal function in a short period of time and is a common pathophysiological process following renal transplantation [1]. AKI is associated with high morbidity and mortality and can progress to chronic renal failure [2,3]. According to an epidemiological survey published in 2015, the incidence of AKI was reported to be 22% in adults and 14% in children [4]. In China, 1.7 million new cases of AKI are reported each year, and 700,000 deaths due to AKI occur per year [5]. The pathogenesis and progression of AKI is complicated, and several risk factors have been implicated, including renal tubular injury, hemodynamic changes, and inflammation [6,7].

The most commonly used animal models of AKI involve the use of ischemia-reperfusion injury, endotoxemia, and drug-induced renal injury, which have all shown that an inflammatory response is associated with AKI [8]. Nuclear factor-κB (NF-κB) signaling is an important part of the innate immune signaling pathway, which is also involved in several key life processes, including inflammation and cell apoptosis [9–12]. Previously reported studies have shown that the suppression of the NF-κB signaling pathway attenuates induced acute renal injury, for example with lipopolysaccharide (LPS), and this signaling pathway may be relevant to the generation of AKI [13–17].

The enhancer of zeste homolog 1 (EZH1) gene, a histone lysine methyltransferase, is the first cloned mammalian gene that is homologous to the *Drosophila melanogaster* enhancer of zeste gene [18]. The EZH1 gene consists of 747 amino acids and contains domains of EZH2_WD binding, SANT, NLS, and SET. The expression of EZH2 is high in mammalian embryos, but very low in adult mammalian tissues, being mainly expressed in organs such as the spleen [18]. The EZH1 gene can interact with the other two core subunits of the polycomb repressive complex (PRC) 2, SUZ12, and EED, to form a PRC2-EZH1 complex with histone H3K27 methylase activity [19].

Current research on the function of the EZH1 gene has mainly focused on cell development and cell differentiation. In the process of myocyte differentiation, the expression of the EZH2 genes has been shown to gradually increase during differentiation, and EZH1 has been shown to directly bind to the genes of myocyte differentiation specific transcription factors, MYOG and MYH to induce the expression of these genes, thereby promoting the normal differentiation of myocytes the polycomb group protein [20]. Ezh1 has been shown to be highly conserved, and the EZH1 gene has importance in the developmental of myocytes [21].

Previously published studies have shown that many histone modifications are involved in regulating the NF-κB signaling pathway. Histone-modifying enzymes regulate the NF-κB signaling pathway in two ways, by modifying the histones on the NF-κB target gene [22], and by modifying the key node proteins of the NF-κB signaling pathway [23, 24]. Saccani and Natoli reported that with the activation of the NF-κB signaling pathway, the level of histone modification of the chromatin of the NF-κB target genes changed significantly, especially the methylation of histone H3K9 and the level of histone acetylation [25]. The EZH1/SUZ12 complex has been shown to regulate the transcription of NF-κB target genes [26]. The transcriptional activity NF-κB Set9 mediated methylation of p65 has been shown to be required for the expression of a subset of NF-κB target genes in response to tumor necrosis factor-α (TNF-α) stimulation [27].

Therefore, the aims of this study were to investigate the effect of overexpression of the EZH1 gene on aristolochic acid-induced injury in HK-2 human kidney proximal tubule epithelial cells *in vitro* and to determine the role of NF-κB signaling.

Material and Methods

Cell culture and an aristolochic acid-induced model of acute kidney injury (AKI)

The human renal tubular epithelial cell line, HK-2, was obtained from Jining Shiye (Shanghai, China). Cells were cultured at 37°C and 5% CO_2 in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, ThermoFisher, Waltham, MA, USA) and the culture media was changed every other day [28].

RPMI medium containing 10% FBS was added with different concentrations (30, 60, and 120 μM/L) of aristolochic acid for 12, 24, and 48h. When the density of HK-2 cells reached 70–90%, the test groups were replaced with the medium containing the corresponding concentrations of aristolochic acid, The control group (untreated group) had only added cell culture medium. The cells continued to be cultured under the culture conditions for another 24 h, and the cells were collected for subsequent processing.

Cell counting kit-8 (CCK-8) assay

HK-2 cells were seeded in 96-well plates and treated with aristolochic acid. Then, 10 μl is CCK-8 medium was added to cells for an additional 2 hours at 37°C in a humidified atmosphere containing 5% CO_2. The optical density (OD) was measured at a wavelength of 450 nm (ThermoFisher, Waltham, MA, USA).

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Cell transfection

Overexpression of the EZH1 and empty control plasmids were purchased from Sino Biological Inc. (Beijing, China). HK-2 cells were seeded into six-well plates (1.0×10^5) for 24 h before transfection and divided into three groups, including the control group (0.1% PBS), the NC group, and the EZH1 group containing the overexpression plasmid. Transient transfection was performed by lipofectamine 3000 (Invitrogen, San Mateo, CA, USA) according to the manufacturer's protocol. A total of 20 μM of overexpressed RNA, control, NC, and 5 μL lipofectamine 3000 were added to the serum-free medium and incubated at 25°C for 10 min. Then, lipofectamine 3000 was mixed into each group and cultured in serum-free RPMI 1640 medium. After 6 h in culture, the fluid was changed back to RPMI 1640 medium containing 10% FBS. The following groups included the control (NC) group, the EZH1 group, the aristolochic acid-treated (AA) group, the NC + AA group, and the EZH1 + AA group.

Flow cytometry

Cells apoptosis was measured using an Annexin-V conjugated with fluorescein isothiocyanate (FITC) kit to label phosphatidylserine sites on the membrane surface (Dojindo Laboratories, Shanghai, China) by flow cytometry. Cells were washed twice using buffer, and the suspension was cultured with Annexin-V FITC and propidium iodide (PI) (Yeasen Biotechnology Co., Ltd, Shanghai, China) in the dark at 25°C for 15 min. Binding buffer was added to each well and the samples were analyzed by flow cytometry within one hour.

The intracellular reactive oxygen species (ROS) levels were measured using a flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Sigma-Aldrich, St Louis, MO, USA). Cells were seeded in a six-well plate. When 80% cell confluence was reached, the cells were exposed to the reagents for 24 hours and 10 μM DCFDA containing 1 ml phosphate buffer saline (PBS) were added to the cells for 20 min at room temperature, and the resulting 2',7'-dichlorodihydrofluorescein (DCF) fluorescence was measured by flow cytometry [29].

Quantitative real-time polymerase chain reaction (qRT-PCR)

The EZH1 and pro-inflammatory cytokine genes (for IL-1β, IL-6, and TNF-α), apoptosis-related genes (BAX, BCL-2, and CYT C), and genes regulating the inflammatory response (NFKBIA, CXCL8, and Cyclin D1) were measured using quantitative real-time polymerase chain reaction (qRT-PCR) in the different groups.

The total RNA was isolated from cultured cells with using TRIzol (Invitrogen, San Mateo, CA, USA) according to manufacturer's instructions. Extracted RNA was reverse transcribed with PrimeScript™ RT reagent kit (TaKaRa, Otsu, Shiga, Japan), performed at 40°C for 45 min and 90°C for 2 min. Then, cDNA was used for amplification using the SYBR Fast qPCR mix (Invitrogen, San Mateo, CA, USA). The PCR reaction consisted of 94°C for 5 min, followed by 45 cycles of 94°C for another 15 s, 60°C for 15 s, and 72°C for 20 s. All primers were purchased commercially (Eurogentec, the Netherlands). The primer sequences are summarized in Table 1. Amplified products were electrophoresed through 2% agarose gels. The amount of RNA was calculated using the $2^{-\Delta\Delta CT}$ method and GAPDH served as an internal control [30].

Table 1. Primers used in quantitative real-time polymerase chain reaction (qRT-PCR).

Gene	Primer	Sequence
EZH1	Forward	5'-CGAGTCTTCCACGGCACCTA-3'
	Reverse	5'-GCAAACTGAAAGACCTGCTTGC-3'
IL-1β	Forward	5'-ACAGAATGAAGCACATCAAACC-3'
	Reverse	5'-ACAGAATGAAGCACATCAAACC-3'
IL-6	Forward	5'-CCCACCGGGAACGAAAGAGA-3'
	Reverse	5'-GCAGGCAACACCAGGAGCAG -3'
TNF-α	Forward	5'-GCCTGCTGCACTTTGGAGTG-3'
	Reverse	5'-TCGGGGTTCGAGAAGATGAT-3'
Bax	Forward	5'-AAGCTGAGCGAGTGTCTCAAG-3'
	Reverse	5'-CAAAGTAGAAAAGGGCGACAAC-3'
Bcl-2	Forward	5'-ATGTGTGTGGAGAGCGTCAAC-3'
	Reverse	5'-AGCAGCCAGGAGAAATCAAAC-3'
Cyt C	Forward	5'-GTCCGGTTGCGCTTTCCTT-3'
	Reverse	5'-CGCAGTTTCCTCAAATTCTTTCTTC-3'
NFKBIA	Forward	5'-ATGGAAGTCATTGGTCAGGTG-3'
	Reverse	5'-ACAGGCAAGATGTAGAGGGGTA-3'
CXCL8	Forward	5'-GCCAACACAGAAATTATTGT-3'
	Reverse	5'-CTGATTCTTGGATACCACAG-3'
Cyclin D1	Forward	5'-TGAACTACCTGGACCGCT-3'
	Reverse	5'-GCCTCTGGCATTTTGGAG-3'
GAPDH	Forward	5'-AGGTCGGTGTGAACGGATTTG-3'
	Reverse	5'-TGTAGACCATGTAGTTGAGGTCA-3'

Western blot

Total protein was extracted from cultured cells using lysis buffer, with RIPA buffer added to each well at 4°C, and centrifuged at 12,000×g for 15min. The bicinchoninic acid (BCA) protein quantification assay (Thermo Fisher, Massachusetts, USA) was used to

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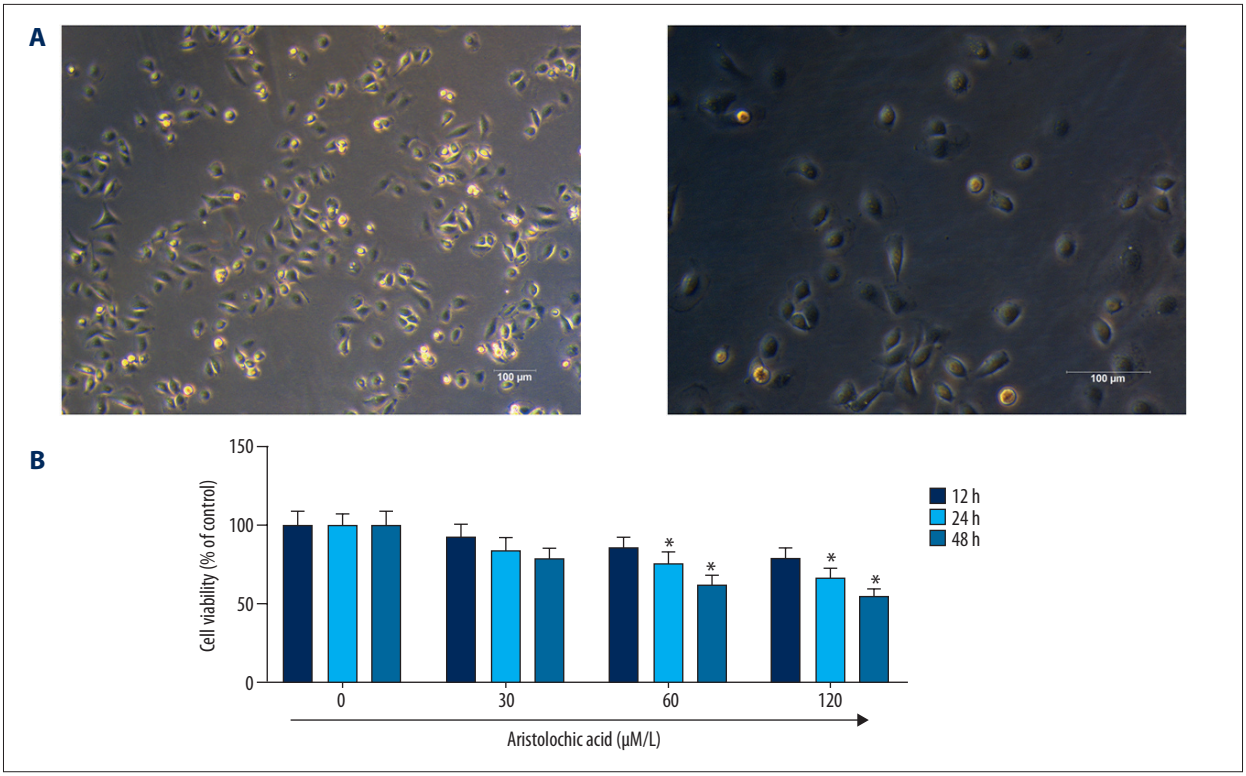

Figure 1. Effects of different concentrations of aristolochic acid on cell injury in HK-2 human kidney proximal tubule epithelial cells *in vitro*. (**A**) The morphology of cultured HK-2 cells were observed under inverted microscopy (100 µm). (**B**) The effects of HK-2 cell injury following administration of different concentrations of aristolochic acid (AA) (30, 60, 120 µM/L) at different times (12, 24, 48 h). Cell viability was determined by the cell counting kit-8 (CCK-8) assay. Data were expressed as the mean ±SD from three independent experiments (* compared with the control). (* P<0.05).

determine the protein content. A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was loaded with protein. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane and blots were blocked with 1% dried milk powder, TBS, and 0.1% Tween-20. Proteins were incubated with primary antibodies including: rabbit anti-EZH1 antibody (1: 1000) (ab64850, Abcam, Cambridge, MA, USA), anti-IL-1β antibody (1: 1000) (ab226918, Abcam, Cambridge, MA, USA), anti-IL-6 (1: 1000) (5145-100, BioVision, USA), anti-TNF-α (1: 1000) (ab6671, Abcam, Cambridge, MA, USA); anti-Bcl-2 (1: 1000) (ab32124, Abcam, Cambridge, MA, USA), anti-Bax (1: 1000) (ab32503, Abcam, Cambridge, MA, USA), anti-Cyt C (1: 5000) (ab133504, Abcam, Cambridge, MA, USA); anti-NFKBIA antibody (1: 2000) (ab7217, Abcam, Cambridge, MA, USA), anti-CXCL8 antibody (1: 10) (ab7747, Abcam, Cambridge, MA, USA), anti-Cyclin D1 antibody (1: 1000) (ab40754, Abcam, Cambridge, MA, USA), washed with TBST, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (ProteinTech, Chicago, Ill, USA) as secondary antibodies. The blot was visualized via enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Inc.). An electrochemiluminescence (ECL) system (GE Healthcare, Chicago, IL, USA) was used to detect the blots. The density of the blots was using Quantity One software version 2.4 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using Prism GraphPad version 6.0 software. All data were presented as the mean ± standard deviation (SD). Differences were compared using one-way analysis of variance (ANOVA) following Turkey's multiple comparison method. A p-value <0.05 was considered to be significant.

Results

Morphological findings in cultured HK-2 human kidney proximal tubule epithelial cells

Following culture of inoculation of the HK-2 cells for 24 h, the growth of adherent cells was observed using an inverted microscope (Figure 1A). The cells were irregular and fusiform and showed rapid growth, and there was no fusion between the cells.

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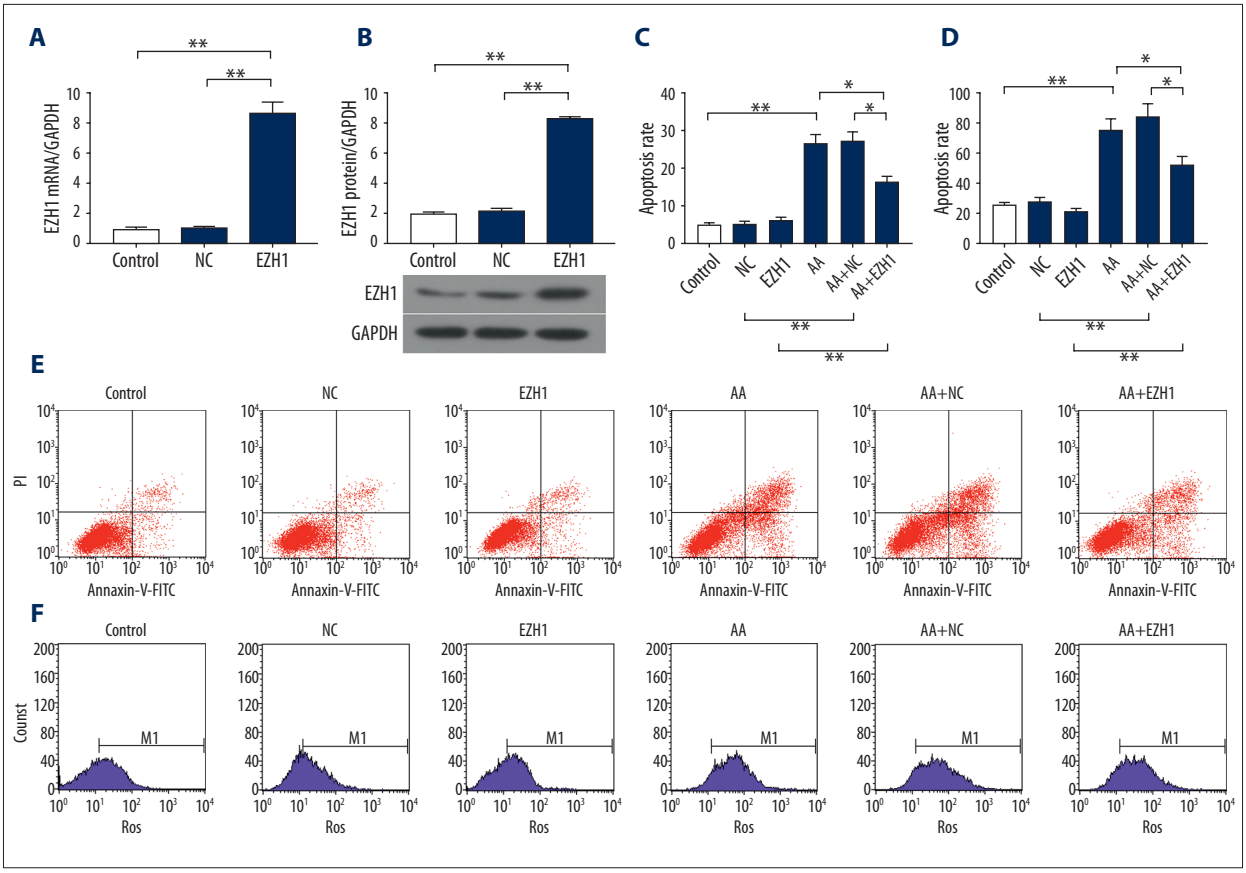

Figure 2. The mRNA and protein expression of the enhancer of zeste homolog 1 (EZH1) gene and the effects of overexpression of EZH1 on aristolochic acid injury-associated apoptosis and reactive oxygen species (ROS) levels in HK-2 human kidney proximal tubule epithelial cells *in vitro*. (**A**) The apoptosis rate and levels of reactive oxygen species (ROS) were analyzed by flow cytometry. The mRNA expression of EZH1 was assessed after transfection by quantitative real-time polymerase chain reaction (qRT-PCR) in three groups of HK-2 cells (control, NC, and EZH1). * P<0.05, ** P<0.01. (**B**) Protein expression of EZH1 was assessed after transfection by Western blot in the three groups of HK-2 cells. * P<0.05, ** P<0.01. (**C**) The relative apoptosis rate was shown as bar diagrams. * P<0.05, ** P<0.01. (**D**) The reactive oxygen species (ROS) were shown as bar diagrams. * P<0.05, ** P<0.01. (**E**) The apoptosis levels were detected by flow cytometry in six groups of HK-2 cells: control, NC, EZH1, aristolochic acid (AA), NC + AA, and EZH1 + AA. (**F**) The ROS levels were detected by flow cytometry in six groups of HK-2 cells *in vitro*: control, NC, EZH1, aristolochic acid (AA), NC + AA, and EZH1 + AA. GAPDH served as an internal control. Data were expressed as the mean ±SD from three independent experiments.

Effects of different concentrations of aristolochic acid on cell injury in HK-2 human kidney proximal tubule epithelial cells

The effects of cell injury following different concentrations (30, 60, 120 μM/L) and different times of treatment (12, 24, and 48 h) of aristolochic acid in HK-2 cells were determined by the cell counting kit-8 (CCK-8) assay. Aristolochic acid treatment reduced cell viability in a dose-dependent and time-dependent manner (60 μM/L, 24 h) (P<0.05, (Figure 1B). Therefore, the subsequent experiments used doses of 60 μM/L of aristolochic acid.

Expression of the EZH1 gene following plasmid transfection in HK-2 human kidney proximal tubule epithelial cells

To determine the transfection efficiency of EZH1 in HK-2 cells, both mRNA and protein levels were detected. The control and NC cells showed no difference in the mRNA level, but the protein expression levels were significantly increased when compared with the control or NC groups (P<0.01) (Figure 2A, 2B).

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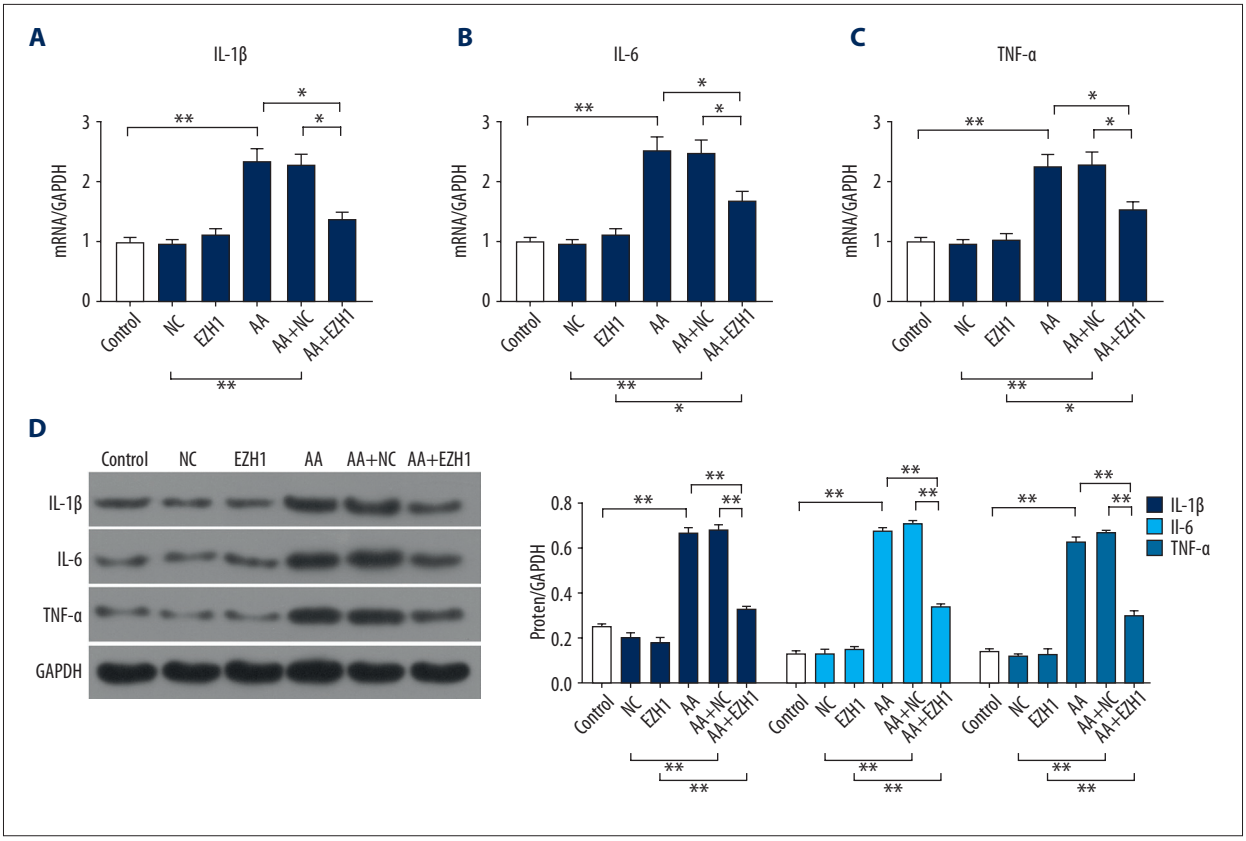

Figure 3. Overexpression of EZH1 affected the expression of inflammatory factors interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) in aristolochic acid injury in HK-2 human kidney proximal tubule epithelial cells *in vitro*. (**A**) The mRNA expression of IL-1β was assessed using quantitative real-time polymerase chain reaction (qRT-PCR) in HK-2 cells. * P<0.05, ** P<0.01. (**B**) The mRNA expression of IL-6 was assessed using qRT-PCR in HK-2 cells. * P<0.05, ** P<0.01. (**C**) The mRNA expression of tumor necrosis factor-α (TNF-α) was assessed using qRT-PCR in HK-2 cells. *P<0.05, **P<0.01. (**D**) The levels of IL-1β, IL-6, and TNF-α protein levels were detected by Western blot in HK-2 cells. * P<0.05, ** P<0.01. GAPDH served as an internal control. Data were expressed as the mean ±SD from three independent experiments.

Overexpression of the EZH1 gene reduced cell apoptosis in aristolochic acid-injured HK-2 human kidney proximal tubule epithelial cells

To assess the effects of overexpression of the EZH1 gene on aristolochic acid injury in HK-2 cells, cell apoptosis was detected using flow cytometry. The findings showed that overexpression of EZH1 showed no clear differences in the rates of apoptosis when compared with the control or NC cells (P>0.05) (Figure 2C, 2E). The single aristolochic acid-treated (AA), NC + AA and EZH1 + AA increased the apoptosis level when compared with the control or NC cells (P<0.01) (Figure 2C, 2E). When aristolochic acid treatment (AA) was combined with overexpression of EZH1, the apoptosis rate was significantly reduced when compared with the aristolochic acid-treated (AA) or NC + AA treated cells (P<0.05) (Figure 2C, 2E).

Overexpression of EZH1 decreased the reactive oxygen species (ROS) level in aristolochic acid-injured HK-2 human kidney proximal tubule epithelial cells

As shown in Figure 2D, 2F, the rapid generation of reactive oxygen species (ROS) by 60 μM/L of aristolochic acid treatment was measured using flow cytometry analysis in HK-2 cells and was compared with the control or NC cells or EZH1 overexpressed cells (P<0.01). In the aristolochic acid-treated cells with overexpression of EZH1, the ROS level was significantly decreased compared with aristolochic acid (AA) or NC + AA cells (P<0.05) (Figure 2D, 2F).

Overexpression of EZH1 decreased the expression levels of inflammatory factors in aristolochic acid-injured HK-2 human kidney proximal tubule epithelial cells

The inflammatory cytokine expression of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) showed similar results

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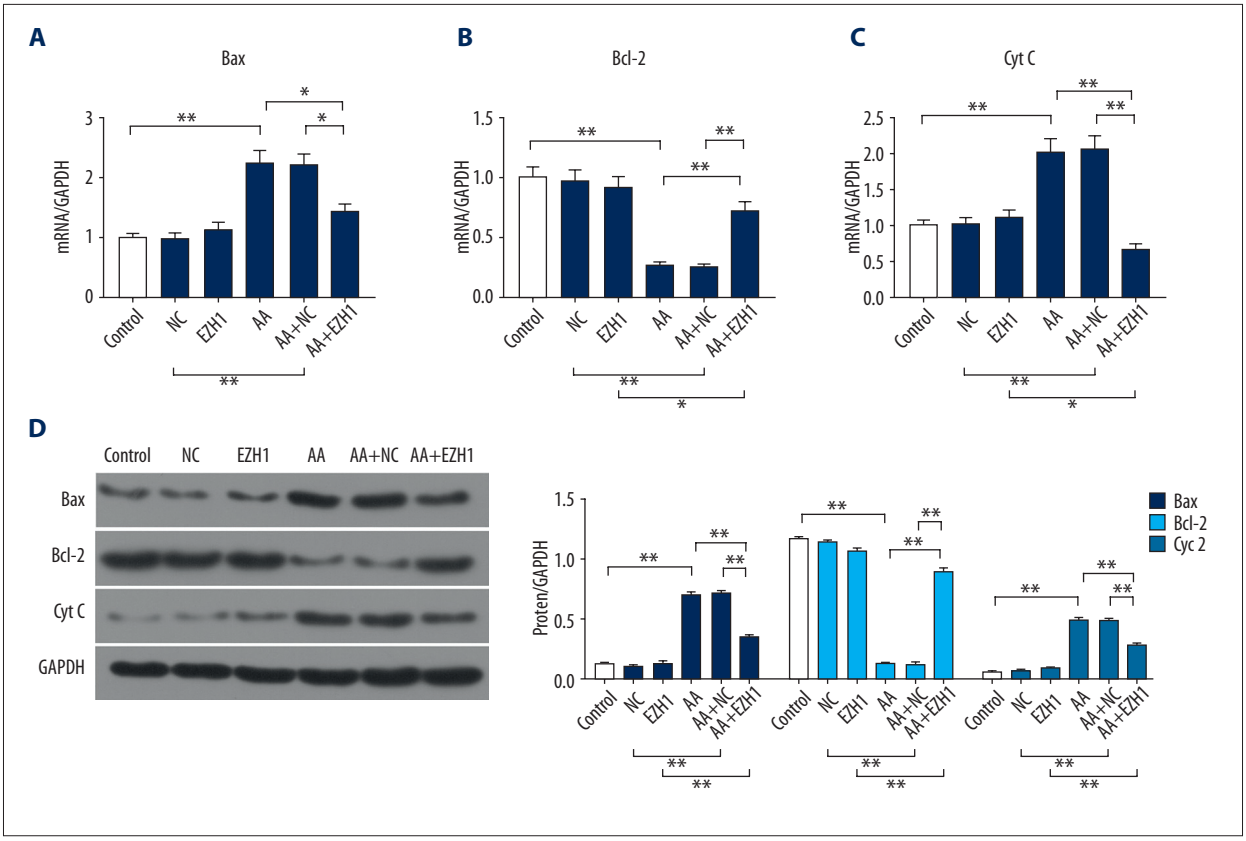

Figure 4. Overexpression of EZH1 affected apoptosis-related genes in aristolochic acid injury in HK-2 human kidney proximal tubule epithelial cells *in vitro*. (**A**) The mRNA expression of BAX was assessed using quantitative real-time polymerase chain reaction (qRT-PCR) in HK-2 cells. * P<0.05, ** P<0.01. (**B**) The mRNA expression of BCL-2 was assessed using qRT-PCR in HK-2 cells. * P<0.05, ** P<0.01. (**C**) The mRNA expression of CYT C was assessed using qRT-PCR in HK-2 cells. * P<0.05, ** P<0.01. (**D**) The Bax, Bcl-2, and Cyt C protein levels was detected by Western blot in HK-2 cells. * P<0.05, ** P<0.01. GAPDH served as an internal control. Data were expressed as the mean ±SD from three independent experiments.

for both mRNA and protein expression levels. The control, NC, and EZH1 gene expression levels determined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot showed that overexpression of EZH1 suppressed NF-κB signaling in aristolochic acid-injured HK-2 cells (Figure 3A–3D). The groups containing aristolochic acid (AA) showed significantly increased expression of IL-1β, IL-6, and TNF-α protein levels compared with the control, NC, or EZH1 cells (P<0.01) (Figure 3D). Compared with aristolochic acid (AA)-treated or NC + AA cells, the EZH1 + AA cells showed down-regulation of protein and mRNA expression of IL-1β, IL-6, and TNF-α (mRNA, P<0.05) (Figure 3A–3C) (protein, P<0.01, Figure 3D).

Overexpression of the EZH1 gene regulated apoptosis-associated gene expression in aristolochic acid-injured HK-2 cells

To detect the effects of overexpression EZH1 on apoptosis, the expression of Bax, Bcl-2 and Cyt C were evaluated using qRT-PCR and Western blot. The Bax and Cyt C showed similar results to that of aristolochic acid-treated (AA) cells. NC + AA cells showed significant upregulation of mRNA and protein levels when compared with the control, NC or EZH1 expressing cells (P<0.01) (Figure 4A, 4C, 4D). The EZH1 + AA group showed down-regulation of the expression of the RNA and protein level of Bax (mRNA, P<0.05) (Figure 4A) protein (P<0.01) (Figure 4D) and Cyt C (P<0.01) (Figure 4C, 4D) compared with AA or NC + AA. The expression of Bcl-2 showed the opposite result to Cyt C in both the RNA and protein level (P<0.01) (Figure 4B, 4D).

Overexpression EZH1 regulated the downstream target genes for NF-κB in both mRNA and protein levels of aristolochic acid-injured HK-2 human kidney proximal tubule epithelial cells

The expression of the NF-κB inhibitor NFKBIA had a similar result with chemotactic factor CXCL8 and Cyclin D1 at both mRNA and protein levels in group control, NC and EZH1. However, aristolochic acid treatment (AA) and NC + AA significantly decreased the expressions of NFKBIA, CXCL 8, and Cyclin D1 mRNA

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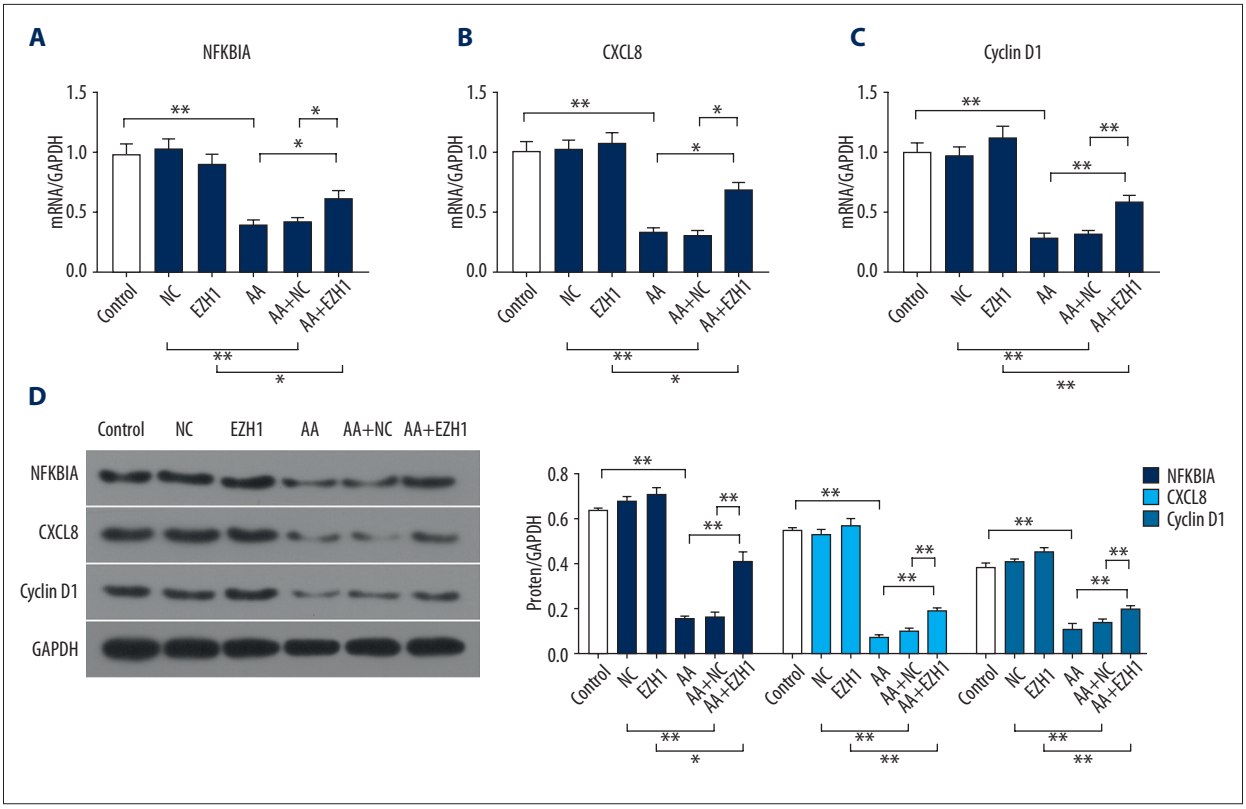

Figure 5. Overexpression of EZH1 regulated the downstream target genes of NF-κB in both the mRNA and the protein levels in aristolochic acid injury in HK-2 human kidney proximal tubule epithelial cells *in vitro*. (**A**) The mRNA expression of NFKBIA was assessed using quantitative real-time polymerase chain reaction (qRT-PCR) in HK-2 cells. * P<0.05, ** P<0.01. (**B**) The mRNA expression of CXCL8 was assessed using qRT-PCR in HK-2 cells. * P<0.05, ** P<0.01. (**C**) The mRNA expression of CYCLIN D1 was assessed using qRT-PCR in HK-2 cells. * P<0.05, ** P<0.01. (**D**) The NFKBIA, CXCL 8, and Cyclin D1 protein levels were detected by Western blot in HK-2 cells. * P<0.05, ** P<0.01. GAPDH served as an internal control. Data were expressed as the mean ±SD from three independent experiments.

and protein levels when compared with the control (P<0.01) (Figure 5A–5D). Compared with the single aristolochic acid-treated (AA) or NC + AA treated cells, EZH1 + AA significantly increased the expression of mRNA of the three genes NFKBIA (P<0.05) (Figure 5A), CXCL 8 (P<0.01) (Figure 5B), and Cyclin D1 (P<0.01) (Figure 5C), with the protein levels were consistent with the mRNA levels (P<0.01) (Figure 5D).

Discussion

Clinically, acute kidney injury (AKI) is a critical disease, due to a variety of causes, such as renal injury and renal ischemia [31]. Previously published studies have shown that an AKI model was successfully established by lipopolysaccharide (LPS) toxicity, rhabdomyolysis-, folic acid and cisplatin toxicity [32–35]. In this study, an *in vitro* model of AKI was established that used different concentrations of aristolochic acid injured HK-2 cells, which showed that 60 μM/L aristolochic acid or aristolochic acid and NC could promote the inhibition of HK-2 cell viability, cell apoptosis, and the reactive oxygen species (ROS) level, and increase the release of inflammatory cytokines and induce NF-κB signaling activation. All of these findings supported that the AKI model was effectively established.

The EZH1 gene is one of the core subfamilies of polycomb repressive complex 2 genes [19]. It has been reported that EZH1 plays an important role in the maintenance and differentiation of stem cells, including embryonic stem cells, hematopoietic stem cells, and epidermal stem cells [36–38]. Recent studies have shown that mutations or abnormal expression of histone modifiers could be detected in many malignant tumors, including the expression of EZH1, which was low in glioblastoma multiforme and high in myeloproliferative neoplasms [39,40]. Also, the EZH1/2 dual inhibitor has shown efficacy against some lymphomas, including multiple myeloma, and leukemia [41,42].

For the first time, in this study, the role of EZH1 involving the acute kidney injury cells was studied. Overexpression of the

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EZH1 plasmid transfected in HK-2 cells showed results that the overexpression of the EZH1 gene reduced apoptosis and ROS levels induced by aristolochic acid in HK-2 cells. Cyt C is the key effector protein from the mitochondrial intermembrane space [43], and together with the anti-apoptosis factor, Bcl-2, and the pro-apoptosis factor, Bax, play an important role in the progression of apoptosis. This study showed that overexpression of the EZH1 gene increased anti-apoptosis Bcl-2 expression and decreased the expression of BAX and CYT C.

Following the occurrence of AKI, renal tubular epithelial cells release a large number of tissue-damaging factors that induce an innate immune response, causing a cascade of amplified inflammatory responses, and producing a large number of pro-inflammatory mediators that include cytokines, chemokines, enzymes, oxygen free radicals, and lipid mediators, further aggravating cells damage or leading to cell necrosis [44,45]. With regard to the release of inflammatory cytokines, silencing of EZH1 could suppress cytokine production, including IL-1β, IL-6, and TNF-α triggered by Toll-like receptors (TLRs) [22]. However, this study showed that the overexpression of EZH1 significantly reduced the release of inflammatory cytokines at the mRNA and protein levels.

Also, in order to understand the protective mechanism underlying the overexpression of EZH1 in aristolochic acid-induced injury to HK-2 cells, NF-κB signaling was expressed that included expressions of the downstream genes, NFKBIA, CXCL 8, and Cyclin D1. It is known that the NF-κB signaling pathway regulates the development of the immune system, the inflammatory response, cell apoptosis, and many other key cellular processes [46–49]. The pro-inflammatory factors, LPS, growth factors, and the antigen receptor-activated IKK complex (including IKKβ, IKKα, and NEMOA), which phosphorylate the IκB proteins (IκBα, IκBβ, IκBε, and Bcl-3), have been shown to lead to IκB protein production, including ubiquitin and lysosomal degradation, resulting in NF-κB release [46,50]. Several molecules have now been shown to be involved in the immune response regulated by NF-κB, including TNF-α, IL-1β, IL-2, IL-6 and IL-8 [50]. Also, NF-κB was has been reported to be associated with the cell cycle, and transcriptionally regulated Cyclin D1 which is involved in the G1/S phase transition [51,52].

The findings of the present study showed that overexpression of the EZH1 gene antagonized the activation of the NF-κB signaling pathway induced by aristolochic acid in HK-2 cells. Specifically, overexpression of EZH1 enhanced the expression of NFKBIA/IκBα in AKI cells, indicating that the NF-κB complex inhibited the release and the expression of some specific genes in response to physiological responses, including cell apoptosis, increased cell injury, and inflammation.

Therefore, the effect of overexpression of the EZH1 gene in aristolochic acid-induced AKI may be partially due to the inhibition of NF-κB signaling. However, the involvement of NF-κB signaling in this study was not fully investigated and remains to be confirmed using a specific activator of NF-κB signaling in future studies.

Conclusions

Overexpression of the enhancer of zeste homolog 1 (EZH1) gene reduced cell injury in HK-2 cell injury induced by aristolochic acid *in vitro* in HK-2 human kidney proximal tubule epithelial cells by inhibition of NF-κB signaling. Overexpression of EZH1 could suppress cell apoptosis, alleviate acute kidney injury (AKI) and reduced the release of inflammatory cytokines.

Conflict of interest

None.

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