Original Article

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.350181

Impact Factor: 1.51

Anthrahydroquinone-2,6-disulfonate alleviates paraquat-induced kidney injury *via* the apelin-APJ pathway in rats

Qi Li[1#], Bo Wang[2#], Kai-Wen Lin[3], Tang Deng[1], Qi-Feng Huang[1], Shuang-Qin Xu[1], Hang-Fei Wang[1], Xin-Xin Wu[1], Nan Li[1], Yang Yi[1], Ji-Chao Peng[1], Yue Huang[1], Jin Qian[1✉], Xiao-Ran Liu[1✉]

[1]*College of Emergency and Trauma, Hainan Medical University, Key Laboratory of Emergency and Trauma of Ministry of Education, The First Affiliated Hospital of Hainan Medical University, Haikou 571199, China*

[2]*Jiangdong Health Center, Meilan District, Haikou 571126, China*

[3]*Hainan Women and Children's Medical Center, Haikou 570312, China*

ABSTRACT

Objective: To explore the protective effects of anthrahydroquinone-2,6-disulfonate (AH_2QDS) on the kidneys of paraquat (PQ) poisoned rats *via* the apelin-APJ pathway.

Methods: Male Sprague Dawley rats were divided into four experimental groups: control, PQ, PQ+sivelestat, and PQ+AH_2QDS. The PQ+sivelestat group served as the positive control group. The model of poisoning was established *via* intragastric treatment with a 20% PQ pesticide solution at 200 mg/kg. Two hours after poisoning, the PQ+sivelestat group was treated with sivelestat, while the PQ+AH_2QDS group was given AH_2QDS. Six rats were selected from each group on the first, third, and seventh days after poisoning and dissected after anesthesia. The PQ content of the kidneys was measured using the sodium disulfite method. Hematoxylin-eosin staining of renal tissues was performed to detect pathological changes. Apelin expression in the renal tissues was detected using immunofluorescence. Western blotting was used to detect the expression levels of the following proteins in the kidney tissues: IL-6, TNF-α, apelin-APJ (the apelin-angiotensin receptor), NF-κB p65, caspase-1, caspase-8, glucose-regulated protein 78 (GRP78), and the C/EBP homologous protein (CHOP). In *in vitro* study, a PQ toxicity model was established using human tubular epithelial cells treated with standard PQ. Twenty-four hours after poisoning, sivelestat and AH_2QDS were administered. The levels of oxidative stress in human renal tubular epithelial cells were assessed using a reactive oxygen species fluorescence probe.

Results: The PQ content in the kidney tissues of the PQ group was higher than that of the PQ+AH_2QDS group. Hematoxylin-eosin staining showed extensive hemorrhage and congestion in the renal parenchyma of the PQ group. Vacuolar degeneration of the renal tubule epithelial cells, deposition of crescent-like red staining material in renal follicles, infiltration by a few inflammatory cells, and a small number of cast formation were also observed. However, these pathological changes were less severe in the PQ+sivelestat group and the PQ+AH_2QDS group (P<0.05). On the third day after poisoning, immunofluorescence assay showed that the level of apelin in the renal tissues was significantly higher in the PQ+AH_2QDS group than in the PQ group. Western blotting analysis results showed that IL-6, TNF-α, NF-κB p65, caspase-1, caspase-8, GRP78, and CHOP protein levels in the PQ group were higher than in the PQ+AH_2QDS group (P<0.05).

Significance

Our study shows that anthrahydroquinone-2,6-disulfonate has a protective effect on paraquat-poisoned kidneys. The mechanism may be linked to reduction in cellular oxidative stress, paraquat content of renal tissue, inflammatory injury, endoplasmic reticulum stress, and apoptosis. Anthrahydroquinone-2,6-disulfonate may play a role in the treatment of paraquat poisoning by upregulating the expression of apelin-APJ.

✉To whom correspondence may be addressed. E-mail: 798052058@qq.com (J. Qian); hy0203049@hainmc.edu.cn (X.R Liu).
#These authors contributed equally to this work.

How to cite this article: Li Q, Wang B, Lin KW, Deng T, Huang QF, Xu SQ, et al. Anthrahydroquinone-2,6-disulfonate alleviates paraquat-induced kidney injury *via* the apelin-APJ pathway in rats. Asian Pac J Trop Biomed 2022; 12(8): 333-342.

Article history: Received 1 March 2022; Revision 13 May 2022; Accepted 16 June 2022; Available online 23 July 2022

The expression of apelin-APJ proteins in the PQ+AH_2QDS group was higher than in the PQ+sivelestat and PQ groups (P<0.05); this difference was significant on Day 3 and Day 7. The level of oxidative stress in the renal tubular epithelial cells of the PQ+AH_2QDS group and the PQ+sivelestat group was significantly lower than in the PQ group (P<0.05).

Conclusions: This study confirms that AH_2QDS has a protective effect on PQ-poisoned kidneys and its positive effect is superior to that of sivelestat. The mechanism of the protective effects of AH_2QDS may be linked to reduction in cellular oxidative stress, PQ content of renal tissue, inflammatory injury, endoplasmic reticulum stress, and apoptosis. AH_2QDS may play a role in the treatment of PQ poisoning by upregulating the expression of the apelin-APJ.

KEYWORDS: Paraquat poisoning; AH_2QDS; Apelin/APJ; Acute kidney injury; Oxidative stress; Rat; Human tubular epithelial cell

1. Introduction

Acute kidney injury (AKI) is a primary manifestation of early damage caused by paraquat (PQ) to the body and is a major cause of the untimely death of patients with paraquat poisoning[1,2]. The mechanism of kidney injury due to PQ is complex and most studies support oxidative stress, inflammatory response, endoplasmic reticulum (ER) stress, and apoptosis as the key factors[3,4]. AKI often presents with acute renal tubular necrosis, vacuolar degeneration of epithelial cells, and renal parenchymal hemorrhage[5,6], resulting in declining kidney function. The kidney is the only organ capable of excreting PQ from the body, and kidney injury leads to delayed PQ excretion, thus aggravating PQ damage to other organs and significantly increasing mortality. Detoxifying drugs and drugs protecting kidney function in the early stages of PQ poisoning are urgently needed in the clinic.

Anthrahydroquinone-2,6-disulfonate (AH_2QDS) has recently been found to have a good detoxification effect on PQ poisoning[7]. It has been reported that AH_2QDS can resist oxidation[8,9]. AH_2QDS has been extensively used in rubber anti-aging agents, metal anti-rust agents, and other industrial applications. In medicine, AH_2QDS is primarily used in cosmetics and the treatment of pigmented skin diseases. Preliminary experiments performed by our research team revealed that AH_2QDS can react with PQ *in vitro* to produce precipitation and reduce PQ concentration in the digestive tract, blood, and urine, thus improving the 30-day survival rate of rats. Lung injury caused by PQ poisoning can be alleviated by reducing oxidative stress and the free radical content in the lungs and inhibiting mild inflammatory reactions. These preliminary experiments also showed that treatment with AH_2QDS reduced the severity of kidney injury and shortened the recovery time for renal function[10,11]. However, the molecular mechanism underpinning the protective effects of AH_2QDS on kidneys remains unclear. Apelin is an endogenous ligand of the G-protein-coupled receptor APJ, a member of the adipokine family, which was initially studied in the cardiovascular system. The apelin-APJ system has been proven to regulate blood pressure and blood glucose and reduce heart injury[12]. In recent years, increasing evidence has emerged that the apelin-APJ system can regulate inflammatory responses in the lungs and kidneys, reduce oxidative stress and apoptosis, and delay aging and progression of diabetic nephropathy[13,14]. However, the role of the apelin-APJ pathway in protection of PQ poisoning using AH_2QDS is rarely discussed. This study aims to explore the effect of AH_2QDS on the kidneys of PQ-poisoned rats and to discuss its protective mechanism.

2. Materials and methods

2.1. Experimental animals and cells

Sprague Dawley rats were purchased from Hunan Changsha Tianqin Biotechnology Co., Ltd. [license: SCXK (Hunan) 2019-0014]. The rats were raised at Laboratory Animal Center, Hainan Medical University with clean grade standard feeding. Human renal tubular epithelial (HK-2) cells were obtained from Shanghai Cell Bank, Chinese Academy of Sciences.

2.2. Main experimental reagents

The primary reagents were as follows: interleukin IL-6 (bs-0782R, Bioss, Beijing, China), tumor necrosis factor TNF-α (bs-10802R, Bioss, Beijing, China), GRP78 (bs-1219R, Bioss, Beijing, China), C/EBP homologous protein (CHOP) primary antibody (bs-20669R, Bioss, Beijing, China); APJ (bs-21310R, Bioss, Beijing, China); nuclear factor-κB (NF-κB) p65 (bs-0465R, Bioss, Beijing, China; ab194726, Abcam, England), caspase-1 (ab286125, Abcam, England), caspase-8 primary antibody (ab25901, Abcam, England); apelin (DF 13350, Affinity, America; orb638328, biorbyt, England); horseradish peroxidase goat anti-rabbit IgG secondary antibody (ab205718, Abcam, England); polyvinylidene fluoride membrane (Biosharp, Beijing, China; Yeasen Biotechnology, Shanghai, China); enhanced chemiluminescence (ECL) hypersensitive colour kit (YEASEN, Shanghai, China); SDS-PAGE gel kit (LR 2107028, Sangon Biotech, Shanghai, China); eosin (Biosharp, Beijing, China); hematoxylin dye solution (Biosharp, Beijing, China); 4', 6-diamidino-2-phenylindole (Beyotime Biotechnology, Shanghai, China); sivelestat (Huilun Jiangsu Pharmaceutical, Shanghai, China); 20% paraquat water agent (Lane Shangqiu Agro-pharmaceutical Factory, Henan, China); standard PQ (AccuStandard, USA); anhydrous sodium carbonate, anhydrous sodium bicarbonate, sodium disulfite (Xilong Scientific, Guangdong, China); AH_2QDS (Chinese

Academy of Tropical Agricultural Sciences Environment and Plant Protection Institute); anthraquinone-2,6,-disodium disulfonate (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan); tricine-SDS-page gel kit (cat: DB208, Little Jumping Frog Biotechnology Co. Ltd., Shanghai, China).

2.3. Main instruments and equipment

Apparatus included gel electrophoresis apparatus (America Bio-Rad Company), analytical balance (Mettler Toledo), fluorescence microscope (Leica, Germany), chemiluminescence fluorescence gel imaging analyzer (Saizhi, Beijing, China), low-temperature centrifuge (Eppendorf, Germany), super-resolution microscope (Olympus, Japan), table type low-speed centrifuge (Hunan Xiangli Scientific Instrument Co. Ltd., Hunan, China), carbon dioxide cell incubator (Shanghai Xinmiao Medical Instrument Manufacturing Co., Ltd., Shanghai, China), inverted fluorescence microscope (Leica, Germany), ultra-low temperature refrigerator (−80 ℃) (Haier, Qingdao, China), rotary slicer (Jinhua Yidi Medical Equipment Co., Ltd., China), ultrapure water system (Merk, Germany), and an autoclave sterilizer (Panasonic, Japan).

2.4. Animal experiment design

Sivelestat is an exogenous neutrophil elastase inhibitor, which has a stable anti-inflammatory effect and can regulate the release of inflammatory factors (*e.g.*, IL-6 and TNF-α), reduce the activation of inflammatory cells, inhibit neutrophil aggregation, decrease inflammatory responses triggered by lung, liver and kidney injury, and protect organ functions[15,16]. So it was selected as the positive control.

A total of 72 Sprague Dawley male rats with an average bodyweight of (300±10) g, were randomly divided into four groups (Control, PQ, PQ+sivelestat, and PQ+AH_2QDS), with 18 rats in each group. The rats fasted for 10 h before being poisoned. To establish poisoning model, 200 mg/kg of 20% PQ solution per rat was administered *via* gavage once based on previous experiment (Supplementary Figure 1). Hematoxylin-eosin (HE) staining confirmed organ damage. For the PQ+sivelestat group, 2 h after PQ poisoning, 30 mg/kg of sivelestat and 1 mL of normal saline per rat were administered *via* intraperitoneal injection once a day for 7 d. For the PQ+AH_2QDS group, 8 mL of AH_2QDS was given intragastrically using a molar ratio of PQ:AH_2QDS = 1:1 (mol:mol) to achieve complete detoxification. From Day 2 to Day 7, the dose was adjusted to 5 mL/day per rat and administered intragastrically. For the control group, PQ group, and PQ+sivelestat group, 8 mL of ultrapure water was given intragastrically on Day 1 after poisoning, and from Day 2 to Day 7, the dose of ultrapure water was adjusted to 5 mL/rat/day once a day *via* gavage. Six rats in each group were collected for tissue examination on Day 1, Day 3, and Day 7 after poisoning. Before dissection, 10% chloral hydrate was injected into the abdominal cavity for anesthesia. Subsequently, 5 mL of blood was collected from the abdominal aorta and centrifuged at 3000 rpm for 5 min. The supernatant was extracted and stored at −80 ℃. The left kidney was excised and placed in 10% neutral formalin. The right kidney was also excised and was placed in a cryopreservation tube and stored in a refrigerator at −80 ℃.

2.5. Measurement of the concentration of PQ in renal tissue

On Day 1, Day 3, and Day 7, the tissues of the right kidneys of each group were washed with ice phosphate-buffered saline (PBS) to clean off blood on the surface of the kidney tissues, and the water was drained using a filter paper. Each specimen of excised kidney tissue was weighed at 100 mg. Subsequently, 500 μL sterile ultrapure water was added to each kidney tissue, which was then homogenized for 3 min at 50 Hz. The specimens were then centrifuged at 12000 rpm for 10 min at 4 ℃, 100 μL supernatant was extracted, and each sample was added to a 96-well plate with a blank control well. The PQ content was measured using the sodium bisulfite-sodium bicarbonate method: 1 g of sodium bisulfite powder, 2 g of sodium bicarbonate powder, and 10 mL of sterile ultrapure water were mixed and centrifuged at 5000 rpm for 10 min twice, the supernatant was extracted and a test solution prepared[17]. To turn on enzyme markers in advance, a 150 μL test solution was promptly added to each sample group (except the blank well), and the OD value (400 nm) was measured immediately. Each rat in each group was tested once (n=6), and the average value for each group was calculated.

2.6. Kidney tissue staining with HE

Kidney tissues were collected from the left kidneys of the specimens from each experimental group at different time points and then fixed in 10% neutral formalin solution for 24 h. These samples were then subjected to gradient alcohol dehydration, transparent xylene, paraffin wax (soft wax and hard wax for 1 h each), embedding, sectioning (0.3 μm), bleaching, baking, dewaxing, hematoxylin staining (6 min), water washing, 0.5% hydrochloric acid ethanol differentiation, water blue (>15 min), eosin staining (7 min), gradient alcohol to water, and sealed sheet. The specimens from each group were then examined for pathological changes, and the degree of tissue damage was scored and recorded. The scoring method was as follows: each of the six rats in each group was examined to select the most suitable pathological slices of dyed renal tissue, and two visual fields in the cortical area and two visual fields in the medulla area were randomly selected for each section (40 ×). For each group, 12 fields of vision in cortex and 12 in medulla area were selected. The areas of the same pathological types in the same field were added and averaged for comparison between groups (Supplementary Table 1).

2.7. Western blotting analysis

On ice, the blood was dried with filter paper and the right kidney tissues of each group were weighed at 50 mg. Then, 500 μL of lysate was added to the sample, which was subsequently subjected to

discontinuous crushing for 3 min at 50 Hz in a tissue homogenizer, ultrasonic cracking at 4℃ for 30 min, and centrifugation at 12000 rpm for 10 min at 4℃, with 400 μL of tissue supernatant extracted. Total protein concentration was measured using a BCA kit and was balanced in each group. A protein loading buffer (1:4, *v/v*) was then added and the denatured protein was boiled for 10 min. Based on the molecular weight and ranking of the protein, different concentrations of sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE) were used; tricine-SDS-PAGE gel was used for the target protein molecular weight <10 kD (apelin). Electrophoresis and membrane transfer was performed at a voltage and duration that corresponded to the molecular weight of the protein, and sealing was done with 5% milk for 2 h. The corresponding protein primary antibody was incubated overnight at 4℃. The resulting film was then washed at 5 min/ounce for half an hour. The corresponding second antibody was incubated at room temperature for 2 h, and the film was washed for half an hour and six times. An assay was developed using an ECL hypersensitive color kit. The grey value was calculated using ImageJ and plotted using GraphPad Prism 8.0. The grey value of the target protein was divided by the grey value of the internal reference (the relative protein expression level) and compared between groups.

2.8. Tissue immunofluorescence

After HE staining and dewaxing, slices were washed three times using cold PBS for 5 min each time. Antigen repair was performed using a pressure cooker, boiling for 5 min. It was then washed with cold PBS three times for 5 min each time and incubated with 3% hydrogen peroxide at room temperature for half an hour. It was washed with cold PBS three times for 5 min each time and sealed with 1% bovine serum albumin solution for half an hour. The corresponding protein was incubated with the primary antibody (1:200, diluted with 1% bovine serum albumin solution), placed in a wet box overnight at 4℃, and then washed with cold PBS three times for 5 min each time. Fluorescein isothiocyanate was added and they were incubated at 37℃ for 1 h and then washed with cold PBS three times for 5 min each time. Then it was stained with a 4', 6-diamidino-2-phenylindole dye core for 5 min in the absence of light, then washed with PBS three times for 5 min each time. An anti-quench agent was then added and the tablet was sealed. Fluorescence microscopy was performed with excitation light of 488 nm and 512 nm.

2.9. Detection of oxidative stress in renal tubular epithelial cells

Human tubular epithelial (HK-2) cells were routinely cultured in an F12-K medium supplemented with fetal bovine serum in a 5% CO_2 incubator at 37℃. The grouping of the HK-2 cells was the same as in the animal experiment. Standard PQ with IC_{50} of 160 μmol/L (Supplementary Figure 2) and cell counting kit 8 (CCK-8) assays were used. Cells from the PQ group, PQ+sivelestat group, and the PQ+AH_2QDS group were treated with 160 μmol/L of PQ for 24 h. After 24 h, cells of the PQ group were moved to a normal medium. The cells from the PQ+sivelestat group were treated with a medium containing sivelestat (100 μg/mL) for 24 h, and the CCK-8 assay showed that it did not affect cell viability. The cells from the PQ+AH_2QDS group were treated with a medium containing AH_2QDS (160 μmol/L) for 24 h, and the CCK-8 assay showed that this did not affect cell viability. After 48 h, oxidative stress levels were measured using reactive oxygen species (ROS) probe kits and photographed with an immunofluorescence microscope at 512 nm.

2.10. Statistical analysis

SPSS Statistics 22 was used for statistical analysis. Fluorescence quantification was analysed using Image J software. Normally distributed measurement data were expressed as mean±SD and analyzed with single-factor analysis of variance (ANOVA). Data with non-normal distribution were expressed as median (IQR) and were tested by Mann-Whitney *U* test. $P<0.05$ was considered statistically significant.

2.11. Ethical statement

The animal protocol was approved by the Ethics Committee of the First Affiliated Hospital of Hainan Medical University [2020 (Research) No. (97)] on July 8, 2020.

3. Results

3.1. AH_2QDS reduces PQ concentration in the kidney

As shown in Figure 1, the PQ content of the PQ group increased significantly compared to the control group ($P<0.01$). The PQ content of the PQ+AH_2QDS group was significantly lower than that of the PQ group on Day 1 and 3 ($P<0.05$). However, the effect of sivelestat was uncertain.

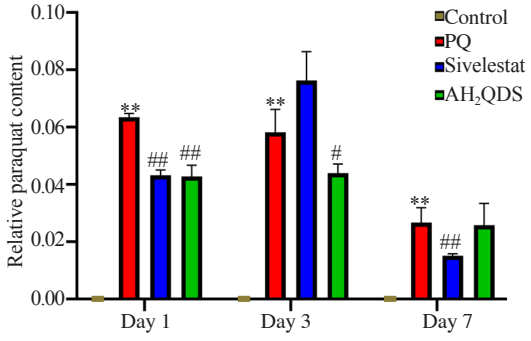

Figure 1. Paraquat (PQ) concentrations in different treatment groups at different times. Sivelestat: PQ+sivelestat group; AH_2QDS: PQ+anthrahydroquinone-2,6-disulfonate (AH_2QDS) group. Compared to the control group, $^{**}P<0.01$; Compared with PQ group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

3.2. AH_2QDS alleviates pathological changes in poisoned kidneys

As Figure 2B shown, on Day 1 after poisoning, renal interstitial hemorrhage and intravascular congestion were more common in medulla area of the PQ group than in the control group. In cortex of PQ group (Figure 2A), there were more crescent-like red deposits in some renal vesicles and glomerular volume increased. The lesions in the PQ+AH_2QDS group were milder than those in the PQ group (P<0.05). On Day 3, renal medulla hemorrhage of the PQ group decreased, but vacuolar degeneration of epithelial cells was obvious in medulla area; in the cortical area, we found a formation of casts. The PQ+AH_2QDS group showed less vacuolation degeneration and hyperemia, and fewer casts were seen (P<0.05). The lesions of PQ+sivelestat were mild (P<0.05). On Day 7 after poisoning, hyperemia in the PQ group was relieved, although there was still obvious epithelial cell degeneration in medulla area, and tubular shape was visible in cortex area; while pathological changes were less obvious in the PQ+AH_2QDS group (P<0.01). Compared with the PQ group, in the PQ+sivelestat group, the pathological damage was reduced (P<0.05) (Figure 2, Supplementary Table 2 and 3).

3.3. AH_2QDS reduces the inflammatory response in kidney tissue

As shown in Figure 3, the expressions of IL-6, NF-κB p65, and

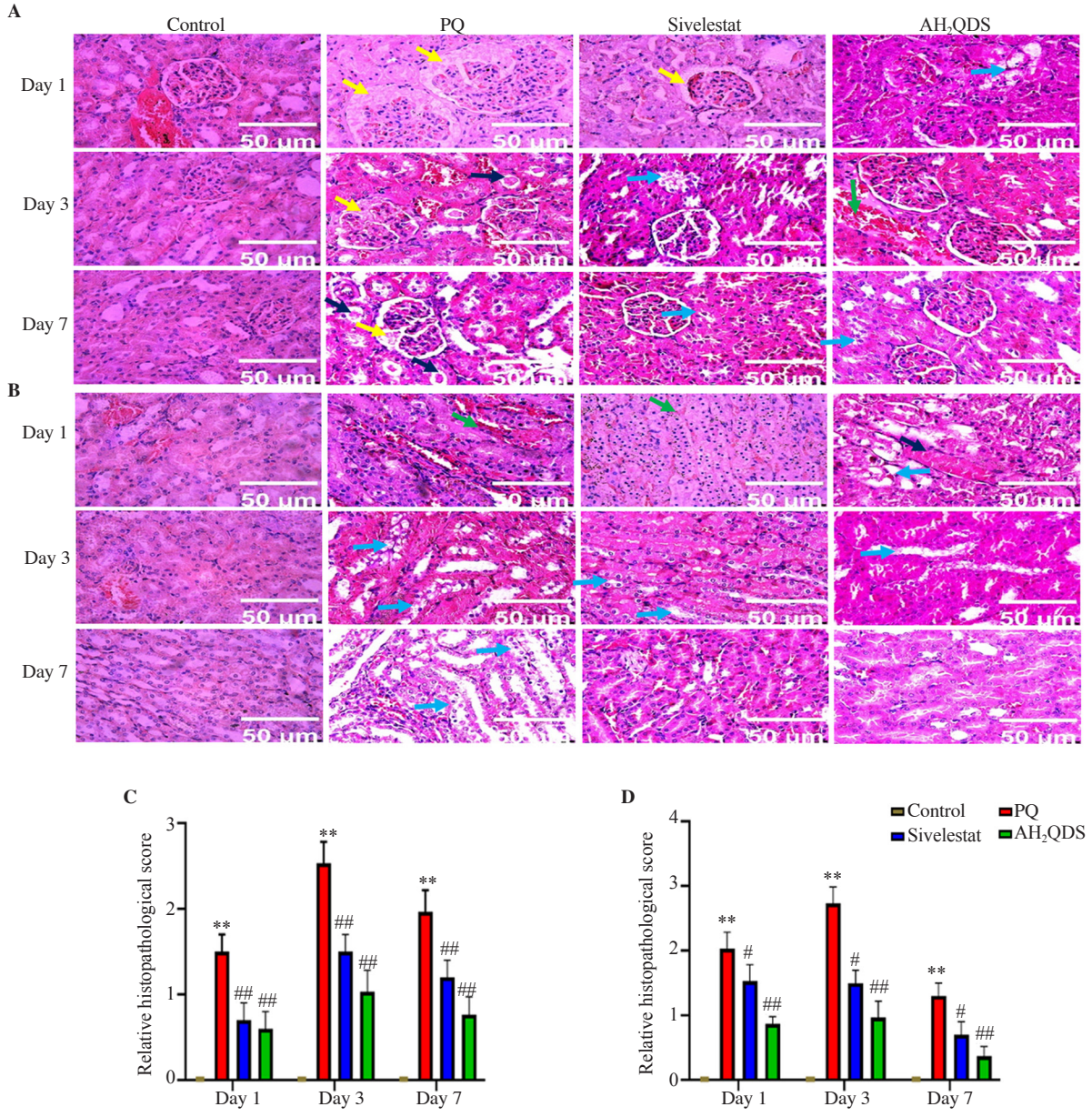

Figure 2. Renal histopathological changes and injury scores. HE-staining of the (A) cortical and (B) medullary regions of the kidney (40×). Renal injury scores in the (C) cortical and (D) medullary regions. Sivelestat: PQ+sivelestat group; AH_2QDS: PQ+anthrahydroquinone-2,6-disulfonate (AH_2QDS) group. Compared to the control group, $^{**}P$<0.01; Compared with the PQ group, $^{\#}P$<0.05, $^{\#\#}P$<0.01. Yellow arrows indicate red stain deposition, green arrows indicate hemorrhage and congestion, blue arrows indicate cavitation degeneration, and black arrows indicate casts.

TNF-α were higher in the PQ group than in the control group (P<0.05). Compared with the PQ group, the expression of IL-6, NF-κB p65, and TNF-α were significantly decreased in the PQ+AH_2QDS group on Day 3 and Day 7 (P<0.01). On Day 1, 3, and 7, IL-6 expression decreased in the PQ+sivelestat group, while NF-κB p65 and TNF-α expression were unstable.

3.4. AH_2QDS reduces oxidative stress

As shown in Figure 4, ROS content in the PQ group was significantly higher than that of the control group (P<0.01). Compared with the PQ group, the PQ+AH_2QDS and PQ+sivelestat groups had lower ROS content (P<0.01).

3.5. AH_2QDS reduces ER stress and apoptosis in renal tissue

As shown in Figure 5, GRP78 expression significantly increased in the PQ group compared with the control group on Day 1 and 3 (P<0.01), and CHOP expression markedly increased on Day 1, 3 and 7 (P<0.01). Moreover, caspase-8 expression increased on Day 3 and 7 (P<0.01). Compared with the PQ group, the expressions of GRP78, CHOP, and caspase-8 decreased gradually on Day 3 and Day 7 in the PQ+AH_2QDS group (P<0.05). In the PQ+sivelestat group, the expressions of CHOP and the caspase-8 protein decreased on Day 1, 3, and 7. No significant decrease in caspase-1 expression was seen in the PQ+sivelestat and PQ+AH_2QDS groups on Day 1 and Day 3, but it was significantly reduced at Day 7 (P<0.01).

3.6. AH_2QDS enhances the expression of apelin and APJ proteins

As shown in Figures 6A, B, and C, apelin expression in the renal tissues of the PQ group decreased on Day 1 and Day 7 compared to the control group (P<0.01), while APJ protein expression decreased

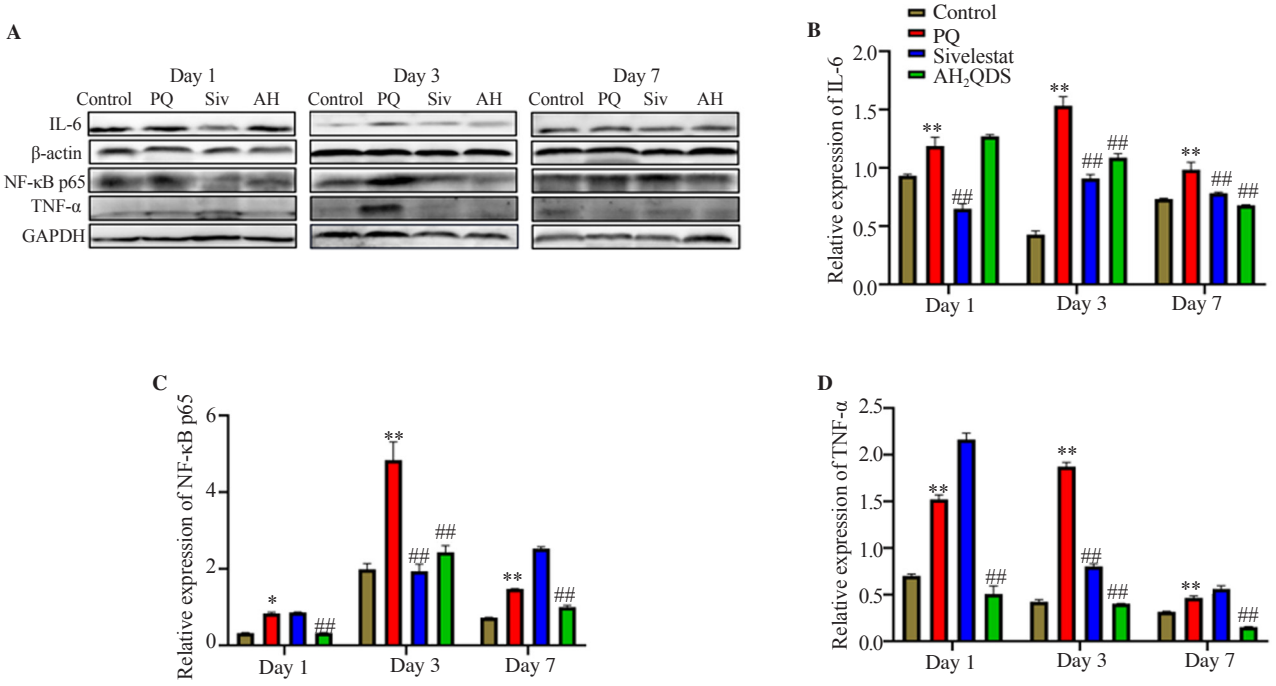

Figure 3. Effect on inflammatory factors. A: Qualitative result; B: Quantitative result of interleukin (IL)-6; C: Quantitative result of nuclear factor-κB (NF-κB) p65; D: Quantitative result of tumor necrosis factor (TNF)-α. Siv/Sivelestat: PQ+sivelestat group; AH/AH_2QDS: PQ+anthrahydroquinone-2,6-disulfonate (AH_2QDS) group. Compared to the control group, $^{*}P$<0.05, $^{**}P$<0.01; Compared with the PQ group, $^{\#\#}P$<0.01.

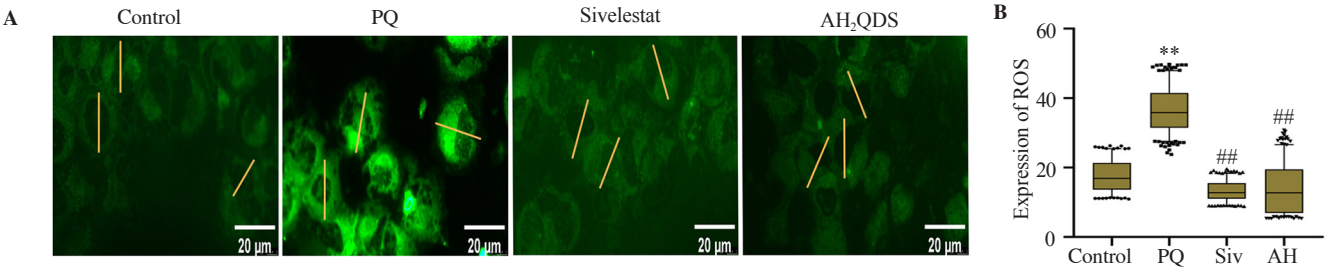

Figure 4. Reactive oxygen species (ROS) levels in human renal tubular epithelial cells. (A) Immunofluorescence of ROS in human renal tubular epithelial cells from different treatments (40×). (B) Quantitative result. Siv/Sivelestat: PQ+sivelestat group; AH/AH_2QDS: PQ+anthrahydroquinone-2,6-disulfonate (AH_2QDS) group. Compared to the control group, $^{**}P$<0.01; Compared with the PQ group, $^{\#\#}P$<0.01. The lines in the figure are fluorescent scanning positions.

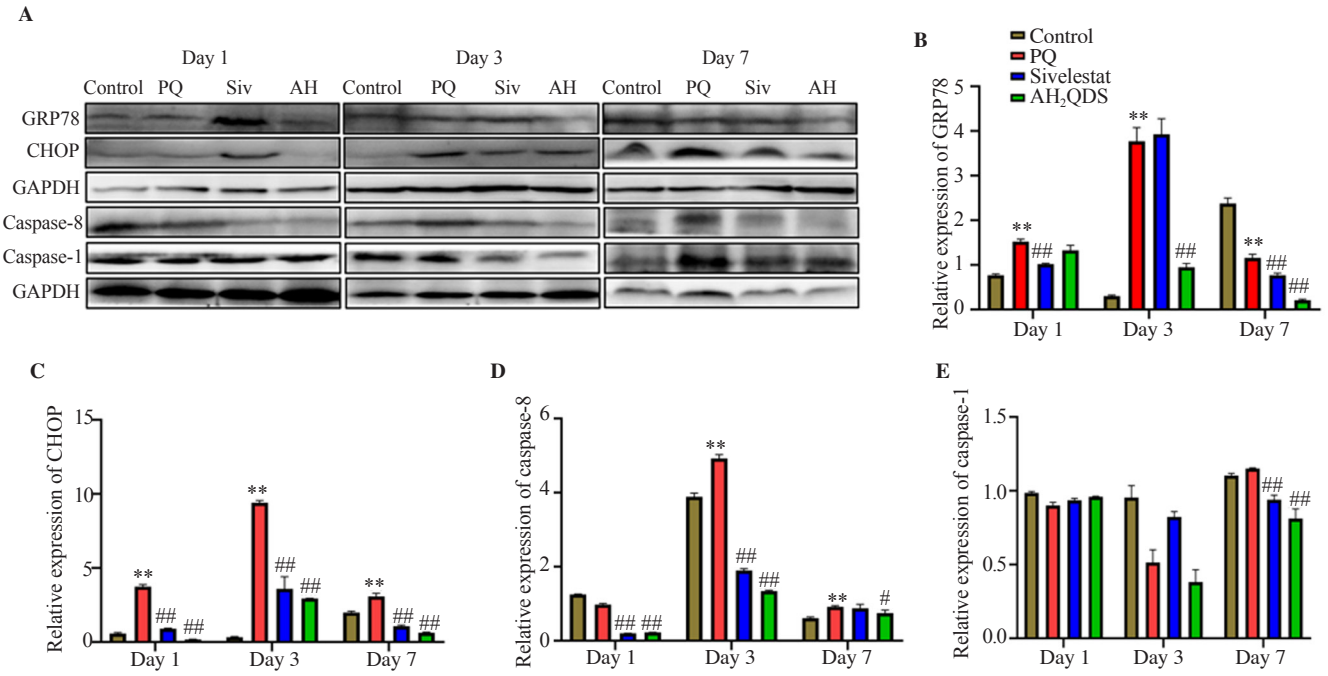

Figure 5. Endoplasmic reticulum stress and apoptotic protein expression in renal tissues. (A) Qualitative result; Quantitative result of (B) GRP78, (C) CHOP, (D) caspase-8, and (E) caspase-1. Siv/Sivelestat: PQ+sivelestat group; AH/AH_2QDS: PQ+anthrahydroquinone-2,6-disulfonate (AH_2QDS) group. Compared to the control group, $^{**}P<0.01$; Compared with the PQ group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

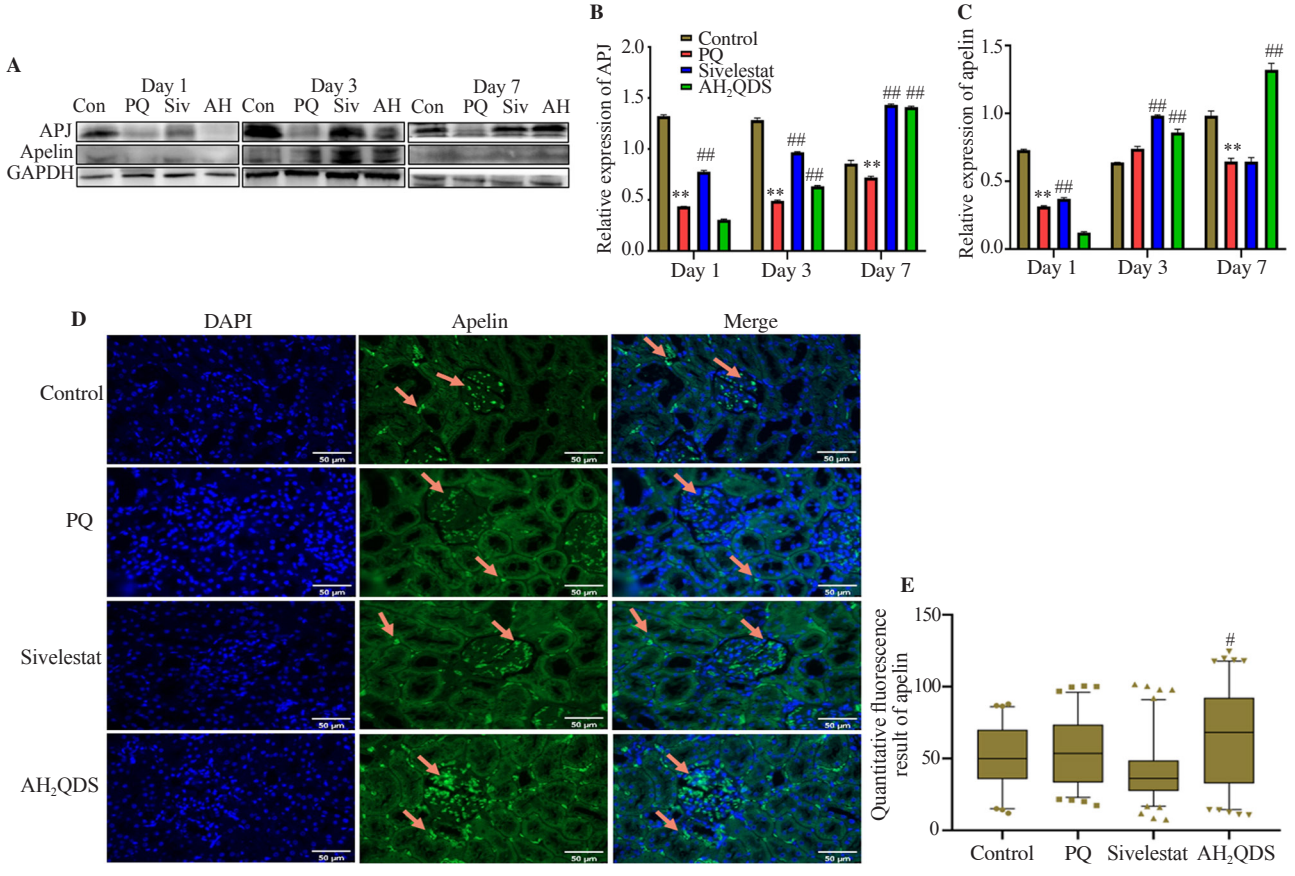

Figure 6. Expression of apelin/APJ protein in the kidney tissue. (A) Western blotting of apelin and APJ protein levels. Quantification result of (B) APJ and (C) apelin in renal tissue. (D) Immunofluorescence of apelin in renal tissue (40×). (E) Quantitative fluorescence result of apelin. Siv/Sivelestat: PQ+sivelestat group; AH/AH_2QDS: PQ+anthrahydroquinone-2,6-disulfonate (AH_2QDS) group. Compared to the control group, $^{**}P<0.01$; Compared with the PQ group, $^{\#}P<0.05$, $^{\#\#}P<0.01$. The arrow shows the region where apelin protein fluoresce strongly.

at three time points (P<0.01). Compared with the PQ group, the expression of apelin and APJ proteins in the PQ+AH_2QDS group increased gradually on Day 3 and Day 7 (P<0.01), whereas in the PQ+sivelestat group, the expression of APJ proteins increased gradually (P<0.01) and apelin expression increased unstably. Figures 6D and E show that apelin fluorescent expression increased in the PQ+AH_2QDS group on Day 3 compared to the PQ group (P<0.05).

4. Discussion

The kidney is the first organ damaged by PQ poisoning, which typically causes degeneration and acute necrosis of renal tubular epithelial cells, renal parenchymal bleeding, congestion, inflammatory cell infiltration, and other pathological changes. It has been reported that PQ poisoning causes a decrease in urine volume and an increase in urine protein content, blood urea nitrogen, creatinine[18], and fluid retention, which causes water-electrolyte imbalance and acid-base balance disorders, further aggravating systemic damage[19,20]. The accumulation of metabolites and toxins aggravates damage to other organs and accelerates the progression of multiple organ dysfunction syndrome.

This study found that PQ content in the kidney of the PQ+AH_2QDS group was significantly lower than that of the PQ group. These results suggest that AH_2QDS can reduce PQ content and alleviate tissue injury. The renal histopathological results on Day 1 show that most of PQ poison converged in the renal cortex of the rat and was accompanied by intravascular bleeding in the glomerular and medulla areas and interstitial blood clots. Furthermore, part of the renal capsule had obvious homogeneous red crescent-shaped dye sediment, significant renal tubular epithelial cell vacuole degeneration, and some lymphocyte infiltration. This is consistent with other reports[21]. In the PQ+sivelestat group and the PQ+AH_2QDS group, renal parenchymal hemorrhage and vacuolation were relatively mild, and renal vesicle deposits were small. In the PQ group, the aforementioned pathological changes were conspicuous on Day 3, and the damage was mitigated on Day 7. Compared with the PQ group, the pathological changes in the PQ+AH_2QDS group and the PQ+sivelestat group were less severe from Day 3 to Day 7. The renal pathology results show that PQ causes inflammatory changes, bleeding, and congestion. Sivelestat and AH_2QDS can mitigate the inflammatory responses of renal tissue and AH_2QDS has a more significant effect. Besides, renal tubular epithelial cells of the PQ group had a higher ROS level, while the PQ+sivelestat group and the PQ+AH_2QDS group had lower ROS levels. PQ causes oxidative stress, while sivelestat and AH_2QDS reduce oxidative stress damage in cells.

Pathological changes in renal tissues may be caused by absorption of PQ into renal circulation or renal tissues, which activates and degranulates lymphocytes and monocytes/macrophages and releases inflammatory factors such as IL-6 and TNF-α[22]. By binding to corresponding target cell surface receptors, these factors may modulate downstream activation of the NF-κB pathway, increasing the expression of inflammatory factors, amplifying the inflammatory response, and triggering an inflammatory storm[23-25]. Eventually, blood vessels dilate, activating the endothelial system, which causes serious fluid and blood cell exudation. Concurrently, this can induce interstitial hemorrhage, edema, degeneration, and necrosis of renal tubular epithelial cells, causing acute kidney tissue damage and resultant deterioration in kidney function. It has been reported that PQ causes oxidative stress, which may be because PQ enters renal tubular epithelial cells. PQ^+ is generated by PQ with mitochondrial nicotinamide adenine dinucleotide phosphate (*i.e.*, reductive coenzyme Ⅱ) and other reductive enzymes. PQ^+ reacts with O_2 to generate PQ^{2+} and $O_2^{\cdot-}$, which produces H_2O_2 under the action of superoxide dismutase. H_2O_2 creates OH^- under the catalysis of Fe, which leads to an increase in ROS content[26]. In addition, reductase depletion leads to redox balance disorders and cellular metabolic disorders. Reductase depletion induces excessive generation of free radicals and destroys membrane structure and transport function, leading to the accumulation of sodium metabolites in intracellular water, which induces cell vacuolar degeneration and necrosis[27]. However, in the PQ+AH_2QDS group and the PQ+sivelestat group, the ROS level was low and there was less tissue damage than in the PQ group. These results indicate that AH_2QDS has a significant antioxidative effect *in vivo* and in cells.

The protein expression of IL-6, TNF-α, NF-κB p65, GRP78, CHOP, caspase-8, and caspase-1 increased to varying degrees in the PQ group, whereas these protein levels were decreased in the PQ+sivelestat group and PQ+AH_2QDS group. These changes were more pronounced from Day 3 to Day 7. These results indicate that sivelestat and AH_2QDS can mitigate these pathological processes to varying degrees and thus protect kidney tissue, and AH_2QDS has a more significant effect. AH_2QDS can reduce oxidative stress and damage due to free radicals, thus reducing the generation of GRP78 and CHOP[28,29]. Furthermore, AH_2QDS could decrease the expression of apoptotic proteins, which is also reported by other researchers[30,31].

It has been reported that apelin-APJ plays a vital role in reducing tissue oxidative stress, inflammatory response, and apoptosis[32-34]. Our results found that compared with the control group, apelin-APJ protein expression decreased in the PQ group. The expression of apelin-APJ proteins was higher in the PQ+sivelestat group and the PQ+AH_2QDS group than in the PQ group and the control group, and the results were more conspicuous from Day 3 to Day 7. Furthermore, we detected immunofluorescence of apelin in renal tissue on Day 3 and found that the distribution density of apelin in the glomerular capillary lobule and interstitial area was higher in the PQ+AH_2QDS group. It indicates that PQ poisoning downregulates the expression of apelin and APJ proteins and possibly weakens the

inhibitory effect of the apelin-APJ system on inflammatory response and oxidative stress. AH_2QDS upregulates the expression of the apelin-APJ pathway and further improves the antioxidative potential of the body[35].

In conclusion, AH_2QDS has a protective effect on PQ-poisoned kidneys. It could inhibit downstream NF-κB activation by upregulating the expression of the apelin-APJ pathway and alleviate inflammatory injury. Besides, AH_2QDS, as an antioxidant, may lower oxidative stress in cells, thereby reducing inflammatory response, ER stress, and apoptosis.

The limitation of this study is that the apelin-APJ pathway involved was not intervened, and it is not clear whether the apelin-APJ pathway is related to Nrf2 and other classical antioxidant pathways, which needs to be further studied. We will use visual labeling technology to study the interactions between pathways.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

It is supported by National Natural Science Foundation of China (No. 81960351); Social Development Key Project of Hainan Province (No. ZDYF2019125); Hainan Provincial Natural Science Foundation of China (820QN398) Hainan Province Clinical Medical Center.

Authors' contributions

QL, BW, JQ, and KWL mainly engaged in experimental design and operation, data collation, article writing, and commissioning. TD, QFH, HFW, and SQX helped the experiment operation, guided the experiment, helped with typesetting, *etc*. XXW, YH, NL, YY, and JCP helped collate data, made diagrams, and modified. XRL helped in experimental design, paper writing and modification, fund support, and submission.

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