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Astragaloside IV attenuates cadmium induced nephrotoxicity in rats by activating Nrf2

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Acute kidney injury (AKI) has become a disease of global concern due to its high morbidity and mortality. This has highlighted the need for renoprotective agents. *Astragaloside IV (AS-IV)* is a saponin isolated from Astragalus membranaceus with good antioxidant, anti-inflammatory and anti-tumor properties. In this study, HK2 cells and rat model were utilized to explore the protective effect of *AS-IV* against cadmium chloride-induced oxidative stress-induced apoptosis. $CdCl_2$-induced apoptosis, ROS production, and mitochondrial membrane potential alterations were significantly inhibited in *AS-IV*-treated HK2 cells. Expression of the mitochondria-associated apoptotic proteins Cleaved-Caspase3, Cleaved-Caspase9, and Cleaved-PARP was significantly reduced after *AS-IV* intervention. In addition, *AS-IV* inhibited Rat weight loss and also alleviated the symptoms of $CdCl_2$-induced nephrotoxicity in a rat model of $CdCl_2$-induced kidney injury. Further experiments showed that *AS-IV* suppresses heavy metal Cd-induced mitochondria-mediated apoptosis by regulating the Nrf2/HO-1 pathway. In conclusion, *AS-IV* could protect the kidney from heavy metal-induced toxicity and could be used as a nephroprotective agent.

Keywords Acute kidney injury, *Astragaloside IV*, Heavy metal cadmium, Oxidative stress, Apoptosis

With the global industrial revolution, pollution of the environment by heavy metals has been increasing, and the production and consumption of heavy metals has also increased dramatically[1]. Cadmium pollution is particularly serious in industries such as nickel-cadmium batteries and pigment[2]. Atmosphere, water and soil are the three main pathways by which cadmium pollutes the environment[3]. Common modes of cadmium ingestion in humans are inhalation through breathing, dermal contact, and ingestion through water[4]. The kidney is the main excretory organ of the body, and cadmium is mainly excreted and retained through the kidney. Therefore, the kidneys have become one of the most serious organs damaged by cadmium[5]. Renal tubular epithelial cells are very sensitive to cadmium exposure. Cadmium-induced damage to renal tubular epithelial cells has been reported to be irreversible, and sustained exposure to cadmium increases the risk of developing total kidney injury[6].

Cadmium as a transition metal mainly causes oxidative damage to tissues. Studies have shown that cellular biomolecules can be catalyzed by transition metals, followed by oxidative reactions, and that excessive accumulation of cellular reactive oxygen species (ROS) destroys their own antioxidant defense system, resulting in oxidative stress[7]. Cadmium ions can cause transient or sustained accumulation of intracellular ROS through several pathways[8]. When cadmium ions enter the cell, the mitochondrial electron respiration transfer chain does not work properly, which leads to the accumulation of cellular ROS[9]. In addition, cadmium can indirectly induce ROS production. Cadmium induces an imbalance in the activity of intracellular antioxidant enzymes and a dysregulation of cellular free radical metabolism, resulting in oxidative stress[10]. For example, MT and GSH bind to cadmium and lose their reductive properties, resulting in the inability to scavenge intracellular ROS[11]. Following oxidative cellular injury, cells initiate a series of signaling pathways that culminate in apoptosis,

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causing AKI[12]. At present, however, chelation therapy for cadmium poisoning is not fully effective in removing cadmium from the body. Therefore, the use of natural antioxidants to reduce tissue damage caused by cadmium poisoning has become a focus of attention[13].

Astragaloside IV (AS-IV) is the main bioactive substances in the traditional Chinese medicine Astragalus membranaceus[14]. *AS-IV* was found to possess antioxidant, anti-inflammatory, immunomodulatory, antiviral and antitumor pharmacological effects. In recent years, *As-IV* has attracted attention in the prevention of renal diseases such as diabetic nephropathy[15], renal fibrosis[16] and launching renal injury[17].However, the pharmacodynamic evaluation and mechanism of *AS-IV* on cadmium metal-induced renal injury are not clear.

In this study, the heavy metal cadmium ($CdCl_2$) was used to model kidney injury. Cadmium-poisoned rats were gavaged with *AS-IV*, and the effectiveness of the drug was assessed by general symptoms, renal function indices and renal histopathology in rat. In in vitro experiments, we co-cultured $CdCl_2$, *AS-IV* with renal epithelial cells and examined the effects of cell viability, ROS changes, apoptosis, and Nrf2/HO-1 pathway protein expression. To explore the mechanism of *AS-IV* anti- $CdCl_2$ nephrotoxicity at the molecular level, it is hoped that this study will provide useful information for the research and development of *AS-IV*-based nutritional products and adjuvant drugs.

Materials and methods

The experiment was conducted in the experimental animal center of Chengdu University of traditional Chinese medicine and submitted to the Institutional Ethics Review Committee for approval (grant no: 2021-47). This study was conducted in strict accordance with the recommendations of the guide for the care and use of laboratory animals issued by the Ministry of science and technology of China. All experiments complied with the ARRIVE.

Reagents and antibodies

Astragaloside IV (purity ≥ 98%, cat: HY-N0431), N-acetyl-L-cysteine (NAC; purity, 99.88%) was purchased from MCE. cadmium chloride was purchased from Sigma (USA, cat: 233-296-7). Antibodies purchased from Proteintect were Nrf2 (China, cat: 16396-1-AP), HO-1 (China, 10701-1-AP), Cleaved-Caspase3 (China 19677-1-AP), Cleaved-Caspase9 (China, 66169-1-Ig), and Cleaved-PARP (China, 15613-1-AP). NQO1 (USA, cat: ab80588) was purchased from Abcam, USA. goat anti-Rabbit IgG Secondary Antibody HRP (China, Cat: A0208) and Goat anti-Mouse IgG Secondary Antibody HRP (China, Cat: A0216) were purchased from Beyotime. GSH assay kit (Beyotime, China, Cat: S0053), MDA assay kit (Beyotime, China, Cat: S0131M) and SOD assay kit (Beyotime, China, Cat: S0101M) were used to assess the level of oxidative stress in fresh kidney homogenates and cultured cell supernatants. ELISA kits were used to detect tumor necrosis factor-α (Elabscience, China, Cat: E-EL-R2856), interleukin-1 β (Elabscience, China, E-EL-RB0013), and interleukin-6 (Elabscience, China, E-HSEL-R0004) supernatant Levels.

Animal models of kidney injury

We purchased 21 SD rats (100 g ± 20) from Chengdu Dashuo Co Ltd (Chengdu, China). All animal experiments were approved by the Animal Ethics Committee of the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine (approval number:2021-47). After 1 week of acclimatization feeding, the rats were randomly divided into three groups: control (Con), *Astragaloside IV* group (AS-IV), $CdCl_2$ group ($CdCl_2$), *Astragaloside IV* low-dose group (AS-IV-L) and *Astragaloside IV* high-dose group (AS-IV-L). According to the previous protocol[18,19], Saline was injected daily intraperitoneally in the control group. 5 mg/kg body weight of CdCl2 was injected daily in the $CdCl_2$ group. 40 mg/kg *Astragaloside IV* intervention was added to the $CdCl_2$ modeling process in the *Astragaloside IV* low-dose group. 80 mg/kg *Astragaloside IV* intervention. Body weight was assessed weekly throughout the experiment. In addition, 24-hour urine samples were collected using separate metabolic cages. 2 weeks later, all rats were sedated and then executed. Serum was collected for biochemical analysis. For pathologic analysis, kidney tissue was immersed in paraffin.

Serum and urine biochemistry

The collected serum and urine were submitted to the Laboratory Department of the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine for the detection of serum urea nitrogen, creatinine, uric acid and 24-hour urinary protein by chemiluminescence.

HE detection

The embedded wax blocks were cut into sections with a thickness of 7 μm. Next, the samples were dewaxed and hydrated with xylene and graded concentrations of alcohol. After hematoxylin staining for 5 min, the sections were stained with eosin for 1 min. The sections were dehydrated using gradient concentrations of alcohol and xylene. Finally, the sections were sealed and the prepared sections were photographed and observed under a 40x lens.

Tunel analysis

Apoptosis was detected and quantified using the TUNEL Brighter Apoptosis Detection Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. The nuclei of apoptotic cells showed green staining, while the nuclei of non-apoptotic cells showed blue color. Fluorescence intensity was detected using a fluorescence microscope (Olympus, Tokyo, Japan).

TNF-α, IL-6, IL-1β assay

A portion of kidney tissue was homogenized with cold saline (1:9) containing 1% protease inhibitor on ice and the homogenate was retained. Levels of pro-inflammatory cytokines including TNF-α, IL-6, IL-1β in the homogenate versus serum were assayed using an ELISA kit according to the manufacturer's instructions.

GSH, MDA, SOD assay

Lipid MDA kit, GSH kit and SOD kit were used according to the manufacturer's instructions to assay GSH, MDA content and SOD activity in kidney tissues of SD rats.

Histological evaluation and immunohistochemical analysis

As previously described[20] assessment of symptoms of cadmium-induced nephrotoxicity. Cadmium-induced nephrotoxicity was evaluated. Rats were intervened as described above, and body weights were assessed every six days. After 2 weeks, all rats were executed, and the kidney tissues were subsequently immersed in paraffin. The embedded wax blocks were cut into sections of 7 μm thickness. Next, the samples were dewaxed and hydrated with xylene and graded concentrations of alcohol. After hematoxylin staining for 5 min, the sections were stained with eosin for 1 min. The sections were dehydrated using gradient concentrations of alcohol and xylene. Finally, the sections were sealed and the prepared sections were photographed and observed under a 40x lens.

Cell culture and cell modeling

Human proximal renal tubular epithelial cells (HK-2 cells) were provided by the Provincial Key Laboratory of the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine. Cells were cultured in DMEM/F-12 containing 10% fetal bovine serum, 1% penicillin and streptomycin and placed in a 37 °C CO2 incubator. $CdCl_2$ modeling was performed when the cell density reached 70–80%. Briefly, the cells were divided into control (Con) group, $CdCl_2$ group and *Astragaloside IV* (0-100 μM) group. Cells in the Control group were placed in a 95% air, 5% CO_2 incubator; cells in the $CdCl_2$ group were incubated in medium supplemented with 30 μM $CdCl_2$ for 24 h. Cells in the *Astragaloside IV* group were treated with different concentrations of *Astragaloside IV* at the same time as $CdCl_2$ (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μM) were incubated for 24 h in total.

Cell viability assay

The protective effect of *AS-IV* was assessed using CCK-8 (Apexbio, USA, cat: K1018). HK-2 cells were co-cultured with $CdCl_2$ in 96-well plates at a density of $1 \times 10\ 4$ cells/mL for 24 h and different concentrations of *AS-IV* were added. finally, 10 μL of CCK-8 reagent was added to each well and the cells were incubated for 1 h. The cells were then incubated with $CdCl_2$ for 1 h. The cells were then incubated for 1 h with CCK-8 reagent. Absorbance was measured at 450 nm by zymography and the experiment was repeated three times.

Flow cytometry

After treating the cells as described above, trypsin was added to digest the cells to obtain a single cell suspension. the supernatant was removed by centrifugation at 300 g for 5 min at 4 °C. The cells were washed with PBS and then centrifuged at 300 g for 5 min at room temperature. The cells were washed with PBS and centrifuged at 300 g for 5 min at room temperature, then the PBS was discarded. the cells were then loaded into sample tubes. The cells were resuspended with 1×Binding Buffer working solution. Subsequently, 5 μL of Annexin V-FITC and 10 μL of PI staining solution were added to each sample tube, gently vortexed, and incubated for 15 min at room temperature away from light, and the apoptosis rate was analyzed using flow cytometry.

ROS levels in cells were measured using the DCFH-DA (Abcam, USA, Cat: ab113851) assay kit. Briefly, single cells were incubated with 10 mM/l DCFH-DA for 30 min at 37 °C. Subsequently, the cells were washed by adding PBS and then centrifuged at 300 g for 5 min at room temperature before discarding the PBS. Finally, cellular reactive oxygen species levels were measured using flow cytometry.

The mitochondrial membrane potential of living cells was determined by JC-1 staining (Beyotime, China CAT: C2006). The intervened cells were aspirated off the original medium and the cells were washed with PBS buffer for 2~3 times. Subsequently, JC-1 staining solution was added, and the cells were incubated at 37 ℃ in an incubator for 30 min, the original staining solution was discarded, and the cells were washed with PBS buffer for 2 to 3 times. Finally, the ratio of cellular mitochondrial membrane potential was analyzed by flow cytometry.

Western blotting (WB)

The kidneys were homogenized in ice-cold RIPA lysis buffer with a mixture of protease and phosphatase inhibitors. The total protein concentration in the supernatant was determined using the BCA Protein Assay Kit. Aliquots of protein samples were separated on SDS-PAGE gels and transferred to PVDF membranes. The membrane was then closed in Tris-buffered saline suspended in 5% skim milk (containing 0.1% Tween 20) for 1 h at room temperature. Next, the PVDF membrane was incubated with the specific primary antibody overnight at 4 °C. After the PVDF membrane was washed with TBST buffer, it was incubated with horseradish peroxidase (HRP)-coupled secondary antibody for 60 min at room temperature. Finally, the immunoreactive signal was detected by chemiluminescence reagent (Biosharp, China, BL520A).

Statistical analysis

All data are presented as mean ± S.E.M. For normally distributed quantitative data, Student's t-test was applied to compare difference between two groups. One-way analysis of variance (ANOVA) and LSD post hoc tests were used to compare difference among three or more groups. For non-normally distributed data, Kruskal-Wallis test was used for comparing difference among different groups.

Result
Effect of *AS-IV* on CdCl$_2$-induced renal injury

Rats were injected intraperitoneally with 5 mg/kg body weight of CdCl$_2$ every other day and treated with *AS-IV* daily. Body weights of the rats were examined daily, and the CdCl$_2$-treated rats showed a significant decrease in body weight, whereas *AS-IV* treatment reversed this trend of weight loss (Fig. 1A). The 24-hour proteinuria, Scr and BUN expression levels were significantly higher in CdCl$_2$-intervened rats compared with the control group.
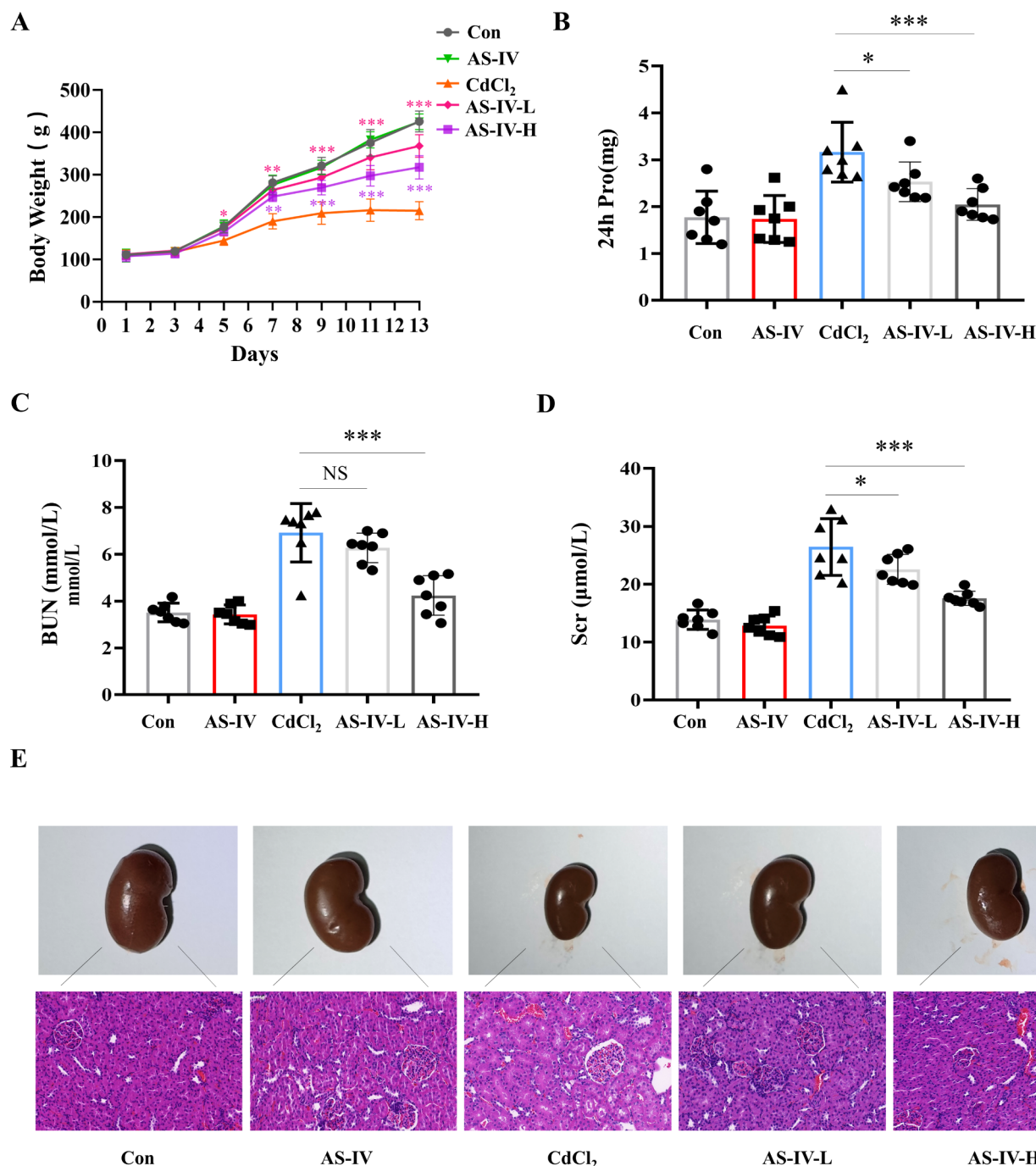
Fig. 1. Effect of *AS-IV* on CdCl$_2$-induced renal injury. (**A**) Changes in body weight of rats ($n = 7$). Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**B**) SCR levels in rats after 14 days. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p = 0.0237$, *AS-IV* -L vs. CdCl$_2$, $p < 0.001$ *AS-IV* -H vs. CdCl$_2$. (**C**) BUN levels in rats after 14 days. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p = 0.0879$, *AS-IV* -L vs. CdCl$_2$, $p < 0.001$ *AS-IV* -H vs. CdCl$_2$. (**D**) 24-hour urine protein levels in rats after 14 days. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p = 0.0276$, *AS-IV* -L vs. CdCl$_2$, $p < 0.001$ *AS-IV* -H vs. CdCl$_2$. (**E**) Morphologic observation and HE staining were used to observe histologic abnormalities in renal tissues. Scale bar, 50 μm.

However, 24-hour proteinuria, Scr and BUN, indicators of kidney injury, were decreased in rats treated with *AS-IV* (Fig. 1B-D). The rats were treated for two weeks and then executed. Subsequently, the gross morphology of the kidneys of the rats was examined. Compared with the control group, the morphology of the kidneys in the $CdCl_2$ group was significantly impaired. It has been shown that cadmium poisoning will induce severe renal tubular injury characterized by tubular dilatation, loss of the tubular brush border, and detachment of tubular epithelial cells[21]. *AS-IV* was found to alleviate renal tubular dilatation with disappearance of tubular brush border by HE staining. In addition, *AS-IV* prevented inflammatory cell infiltration (Fig. 1E).

To further confirm the cadmium-induced renal injury, we examined the nephrotoxicity markers fibronectin, collagen. as expected, the heavy metal cadmium promoted the expression of the nephrotoxicity markers fibronectin, collagen. After *AS-IV* treatment, fibronectin, collagen expression decreased significantly (Fig. 2A, B).

AS-IV reduces $CdCl_2$-induced inflammatory factors. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Two weeks after exposure of rats to cadmium, blood was taken through the abdominal aorta and centrifuged to obtain rat serum. In addition, a portion of kidney tissue was homogenized with cold saline (1:9) containing 1% protease inhibitor on ice, and the homogenate was retained. The pro-inflammatory factors TNF-α, IL-6 and IL-1β were significantly elevated in the $CdCl_2$ group of rats compared to the control group. The concentrations of pro-inflammatory factors TNF-α, IL-6 and IL-1β were significantly decreased in rats treated with *AS-IV* (Fig. 3A-F). It was demonstrated that *AS-IV* significantly inhibited $CdCl_2$-induced renal inflammatory response.

Effect of *AS-IV* on cadmium-induced apoptosis in rat kidney cells

To investigate the effect of *AS-IV* on apoptosis in rat kidney cells, we detected apoptosis in rat kidney tissues using Tunel assay. Apoptotic cells were significantly increased in the cadmium intervention group compared with the control group. However, apoptotic cells were significantly reduced after *AS-IV* treatment compared with the model group (Fig. 4A). Subsequently, the anti-apoptotic effect of *AS-IV* was further verified by Western blot.*AS-IV* blocked the expression of $CdCl_2$-induced apoptotic proteins Cleaved-PARP, Cleaved-Caspase3 and Cleaved-Caspase9(Fig. 4B).

Effect of *AS-IV* on oxidative stress

The effects of oxidative stress on renal injury are wide-ranging and cover all aspects of renal pathology as well as systemic complications[22]. Oxidative stress leads to renal injury and dysfunction by inducing apoptosis, necrosis, and inflammation in renal cells, and further leads to progressive decline in renal function[23].Study finds long-term exposure to cadmium upsets oxidative and antioxidant balance, causing kidney damage. We used ELISA for superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) to assess oxidant product levels. As shown in Fig. 5A-C, MDA increased by approximately 300% and GSH and SOD decreased by 50% and 75%, respectively, in the $CdCl_2$-treated group compared to the control group. When *AS-IV* was treated MDA, GSH & SOD expression levels were restored. To further assess the effect of *AS-IV* on oxidative stress, we measured the expression of oxidative stress-related proteins using WB. *AS-IV* activates the expression levels of Nrf2 and its downstream target proteins HO-1 and NQO1(Fig. 5D).

AS-IV inhibits $CdCl_2$-induced reduction in HK-2 cell viability and apoptosis

As cadmium poisoning will induce severe tubular damage leading to tubular cell apoptosis. To investigate the protective effect of *AS-IV* on the kidney in more detail, Renal tubular cells(HK2)were exposed to different concentrations of $CdCl_2$ and *AS-IV* for 24 h respectively. CCK8 experiments revealed that cell viability decreased by 50% when $CdCl_2$ reached 50 μM (Fig. 6A). HK2 cell growth will be inhibited at *AS-IV* concentrations up to 100 μM (Fig. 6B). Subsequently, HK2 cells were treated with 50 μM $CdCl_2$ to induce apoptosis. The increase in cell viability after treatment with different concentrations of *AS-IV* was dose-dependent (Fig. 6C). To further prove the accuracy of the above results, we performed apoptosis experiments. As shown in Fig. 6D, the apoptosis rate was 30% in the $CdCl_2$ group, and the apoptosis rate decreased to 10% after 70μM *AS-IV* intervention. Subsequently, the anti-apoptotic ability of *AS-IV* was further verified by Western blot.*AS-IV* blocked the expression of $CdCl_2$-induced apoptotic proteins Cleaved-PARP, Cleaved-Caspase3 and Cleaved-Caspase9 (Fig. 6E).

AS-IV inhibits $CdCl_2$-induced ROS production in HK2 cells with altered mitochondrial membrane potential through activation of the Nrf2 pathway

We further investigated the role of oxidative stress in cadmium-induced kidney injury in vitro. Mitochondria play a key role in energy metabolism as well as oxidative stress in kidney cells[24]. I used flow cytometry to determine the mitochondrial membrane potential changes and reactive oxygen species levels. The results showed that the fluorescence of JC-1 polymer had a decrease in intensity and the fluorescence of JC-1 monomer had an increase in intensity after $CdCl_2$ intervention in HK2 cells, resulting in a significant increase in the fluorescence intensity of JC-1 polymer/monomer.*AS-IV* treatment reversed the mitochondrial membrane potential alteration (Fig. 7A, D). Similar to the JC-1 results there, *AS-IV* inhibited $CdCl_2$-induced ROS accumulation (Fig. 7B, C). Subsequently, WB results showed that *AS-IV* was able to activate the expression levels of Nrf2 and its downstream target proteins HO-1 and NQO1 (Fig. 7E).

To further recognize the therapeutic effect of *AS-IV*, we compared *AS-IV* with the antioxidant N-acetyl-L-cysteine (NAC). Subsequently, we co-cultured 35 μM AS-IV, 70 μM *AS-IV*, and 2 mM NAC with HK2, respectively. *AS-IV* was able to inhibit ROS production more effectively than the NAC intervention(Fig. 8A). *AS-IV* had a stronger ability to activate the expression of oxidative stress-related protein Nrf2 and its downstream target proteins HO-1 and NQO1(Fig. 8B). In addition, we examined mitochondrial dynamics and energy
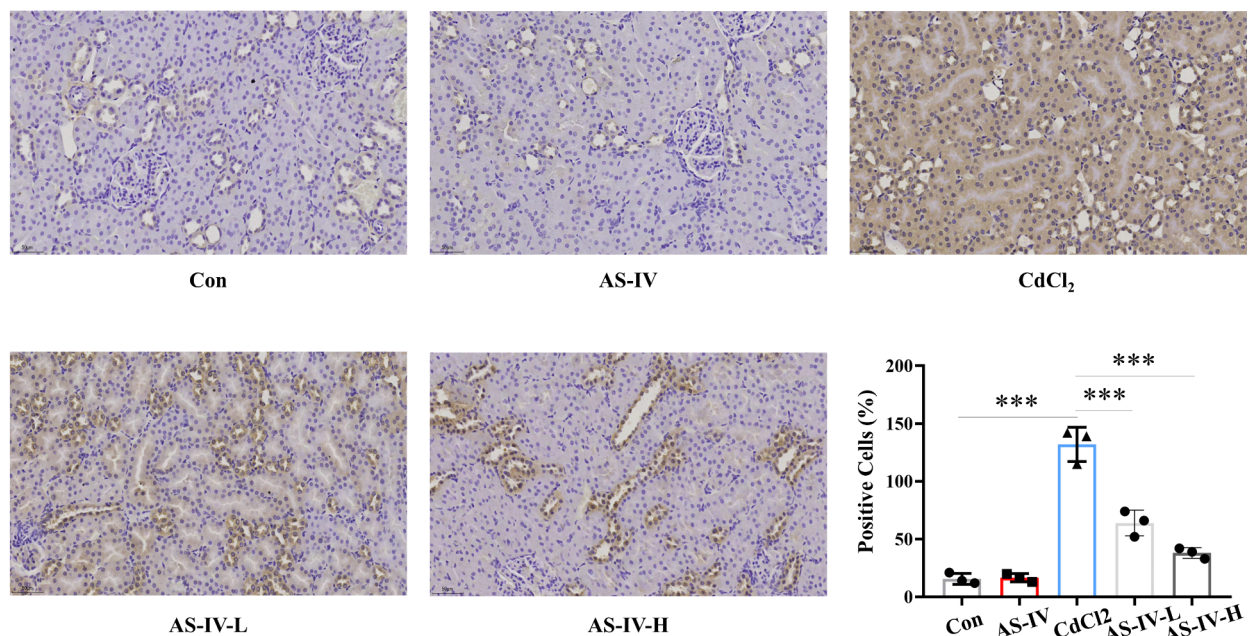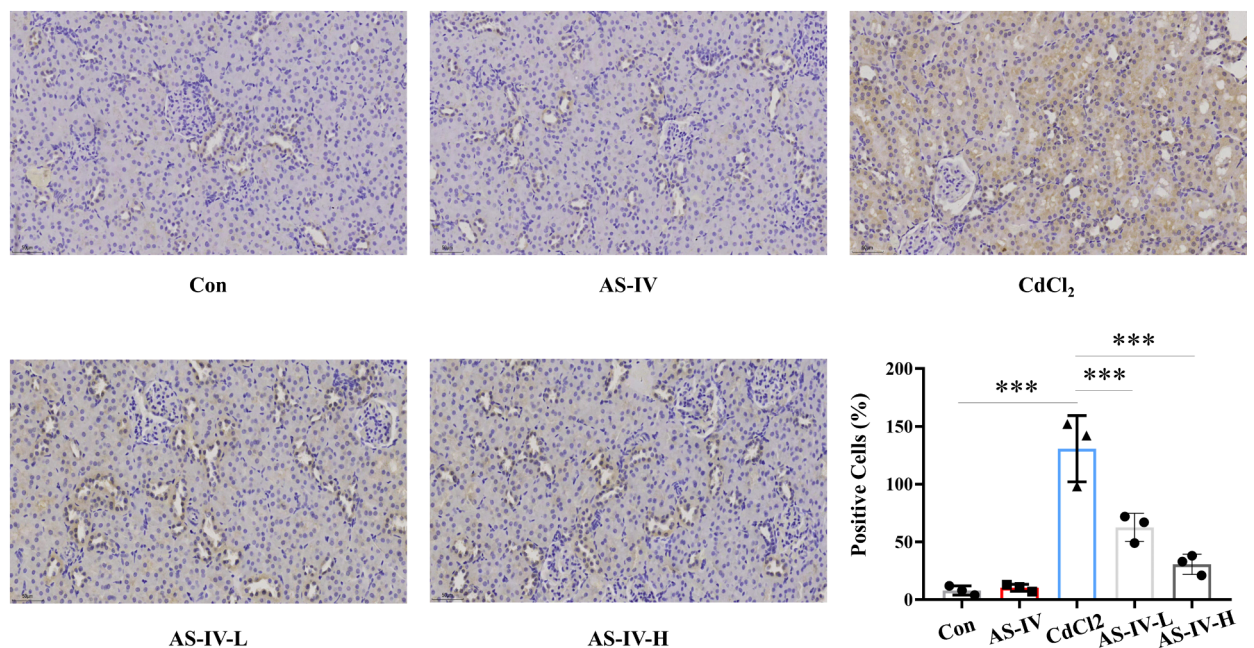
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Fig. 2. *AS-IV* inhibits cadmium-induced nephrotoxicity. (**A**, **B**). Kidney tissues were obtained two weeks after the rats were treated with CdCl2. The kidney fibronectin, collagen expression was observed by immunohistochemistry.

metabolism by an ATP kit. As shown in Fig. 8C, the ATP content, which was inhibited by $CdCl_2$, was effectively protected by *AS-IV* treatment.

Discussion

With the development of economy and industry, heavy metal pollution is of great concern, people can ingest cadmium from the environment and food in the body accumulation, which leads to acute and chronic cadmium poisoning[25].At present, there is no better means of antagonizing cadmium poisoning, when acute ingestion of cadmium poisoning occurs, mainly given a large number of rehydration, injection of atropine antiemetic and elimination of abdominal pain and other symptomatic treatment. Metal chelating agents, such as sodium
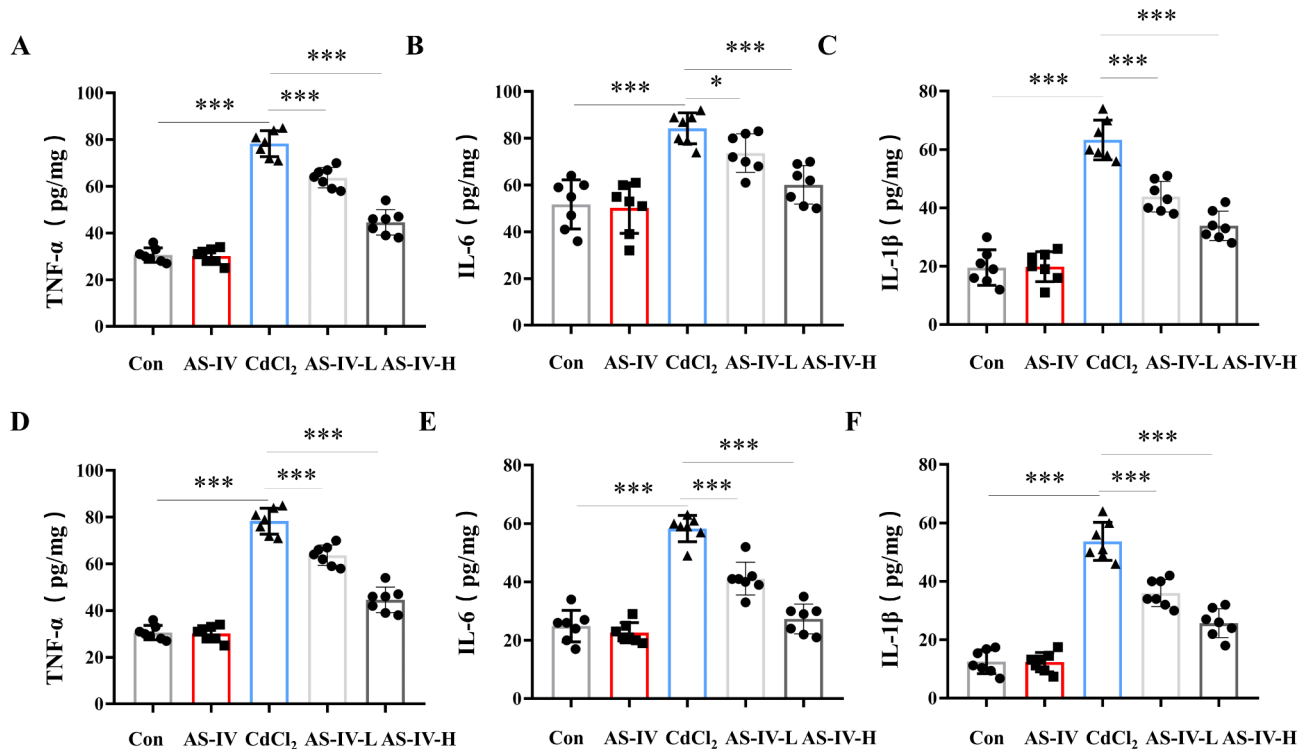
Fig. 3. *AS-IV* reduces CdCl$_2$-induced inflammatory factors. (**A**) Elisa for serum levels of inflammatory cytokines TNF-α. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p < 0.001$ AS-IV -L vs. CdCl$_2$, $p < 0.001$ AS-IV -H vs. CdCl$_2$. (**B**) Elisa for serum levels of inflammatory cytokines IL-6. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p = 0.419$ AS-IV -L vs. CdCl$_2$, $p < 0.001$ AS-IV -H vs. CdCl$_2$. (**C**) Elisa for serum levels of inflammatory cytokines IL-1β. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p < 0.001$ *AS-IV* -L vs. CdCl$_2$, $p < 0.001$ *AS-IV* -H vs. CdCl$_2$. (**D**) Elisa for kidney tissue levels of inflammatory cytokines TNF-α. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p < 0.001$ AS-IV -L vs. CdCl$_2$, $p < 0.001$ AS-IV -H vs. CdCl$_2$. (**E**) Elisa for kidney tissue levels of inflammatory cytokines IL-6. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p < 0.001$ AS-IV -L vs. CdCl$_2$, $p < 0.001$ AS-IV -H vs. CdCl$_2$. (**F**) Elisa for kidney tissue levels of inflammatory cytokines IL-1β. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. CdCl$_2$, $p < 0.001$ AS-IV -L vs. CdCl$_2$, $p < 0.001$ AS-IV -H vs. CdCl$_2$.

edetate, can not antagonize cadmium toxicity, and even aggravate the toxic effect of the possible[26].And it has been found that natural products such as dandelion flavonoids and garlic mixtures are effective in antagonizing cadmium poisoning[27].Therefore, it is important to search for antidotes against cadmium toxicity from natural products.

Astragaloside IV (AS-IV), one of the main active components of Astragalus, is a cycloalkane triterpene glycoside[28]. In recent years, a large number of animal, cell and clinical experiments have shown that Astragalus has anti-inflammatory, antioxidant, anti-apoptotic, anti-fibrotic, anti-tumor, anti-neurological damage, and protection of cardiovascular and cerebral vascular effects, and it can be widely used in the treatment of various immune inflammation, diabetes mellitus, neurovascular diseases and so on[29–31]. Especially in renal disease, it has been confirmed that astragalus found different degrees of protective effects on cisplatin-induced renal injury, adriamycin-induced renal injury, streptozotocin-induced renal injury[32–34]*AS-IV*, as the main active ingredient of Astragalus, has been shown to be useful as a therapeutic or adjunctive agent for acute nephritis. However, the relationship between *AS-IV* and Cd-induced renal injury is not clear. Here, in order to fill the gap in this research field, we established a CdCl$_2$-induced kidney injury rat model and a HK-2 cell model to investigate the possible role and potential mechanism of *AS-IV* in this process.

In this study, the body weight of the rats reflected the physical health of the animals. Long-term exposure to the heavy metal cadmium led to weight loss and the rats were no longer healthy compared to the control group. Since the kidney is the main excretory organ of the body, it is a target organ for cadmium accumulation[35]. Subsequently, I tested renal function markers in rats. The results showed that the kidneys were affected by chromium toxicity, with 2-hour proteinuria, elevated serum CRE and BUN levels, and impaired renal reabsorption and filtration. We dissected the rats and found that the kidneys showed congestion, edema and organ atrophy, etc. HE staining showed that the renal tissues showed massive inflammatory cell infiltration, loss of brush border, obvious formation of tubular pattern and dilatation of renal tubules. Tunel staining results showed that the toxic effects of cadmium were also manifested in apoptosis. Long-term exposure to the heavy metal cadmium leads to renal cell apoptosis. Inflammatory processes often occur throughout chronic kidney
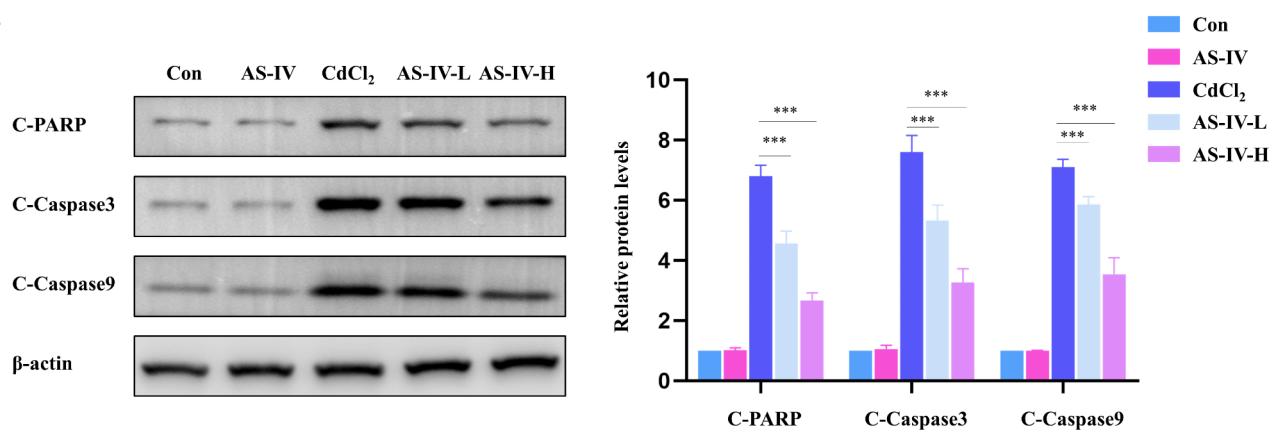
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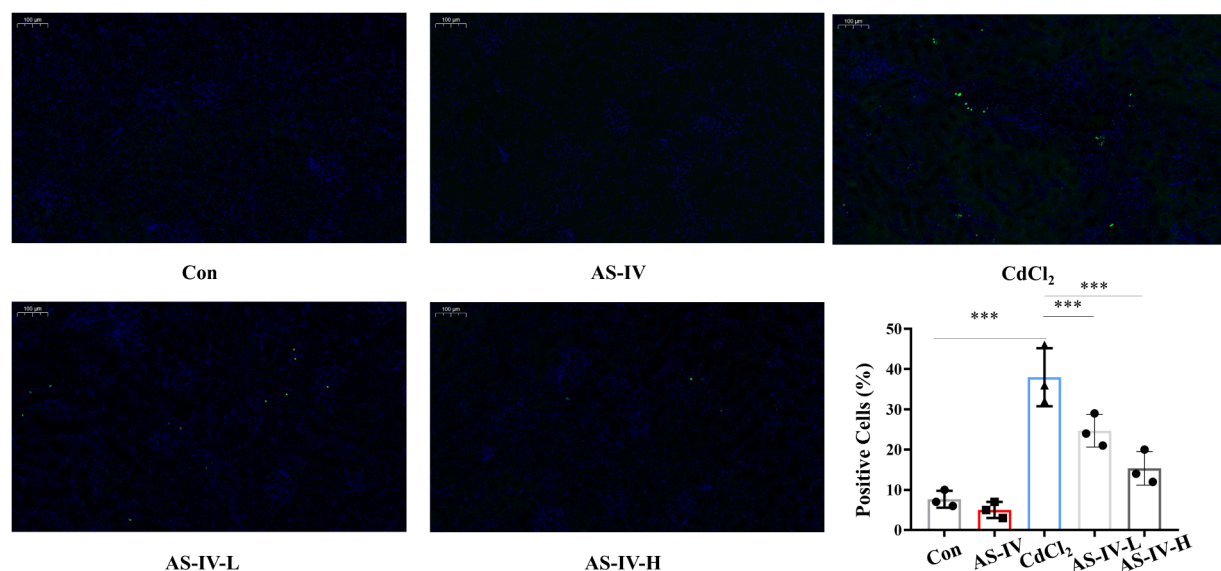
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Fig. 4. *AS-IV* ameliorates cadmium-induced renal cell apoptosis. (**A**) Tunel staining was used to determine the rate of apoptosis in renal tissues. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**B**) Measurement of Cleaved-PARP, Cleaved-Caspase3 and Cleaved-Caspase9 protein expression by WB. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

disease, and the inflammatory response is the body's defense against inflammatory factors, in which the release of inflammatory factors plays a key role[36]. TNF-α, IL-6 and IL-1β were significantly higher in the serum of rats after heavy metal intervention compared to the control group. The above results indicate that the kidneys are susceptible to cadmium toxicity with long-term exposure to the heavy metal cadmium. As expected, when we used *AS-IV* to intervene in cadmium-intoxicated rats, the above deterioration of renal function was significantly improved by *AS-IV*. The improvement of $CdCl_2$-induced acute kidney injury by *AS-IV* was demonstrated from a macroscopic morphologic point of view.

It has been suggested that kidney injury and proximal tubule cell apoptosis may be due to cadmium-induced oxidative stress[37]. However, the underlying mechanisms by which cadmium exposure causes oxidative stress in kidney and proximal tubule cells are unknown. Nrf2 is a typical anti-oxidative stress pathway[38]. We speculate that *AS-IV* significantly enhances the expression of Nrf2 and increases the activities of glutathione, peroxidase, and superoxide dismutase, resulting in cadmium-induced oxidative stress injury in renal tubular epithelial cells. We proved the conjecture by Elisa for SOD, GSH and MDA and WB for Nrf2 pathway proteins. To more fully verify the authenticity of our results. We performed a cellular model of $CdCl_2$-induced HK2 injury in renal tubular cells. Consistent with the results of in vivo experiments *AS-IV* could effectively inhibit $CdCl_2$-induced apoptosis. Notably, when the concentration of *AS-IV* exceeded 100 μM, *AS-IV* not only failed to resist apoptosis, but also promoted apoptosis. This result suggests that a higher dosage of *AS-IV* is not better when *AS-IV* is used clinically for the treatment of acute kidney injury due to Cd. Oxidative stress-related experiments revealed that $CdCl_2$ would inhibit the Nrf2 pathway, inducing cellular reactive oxygen species accumulation
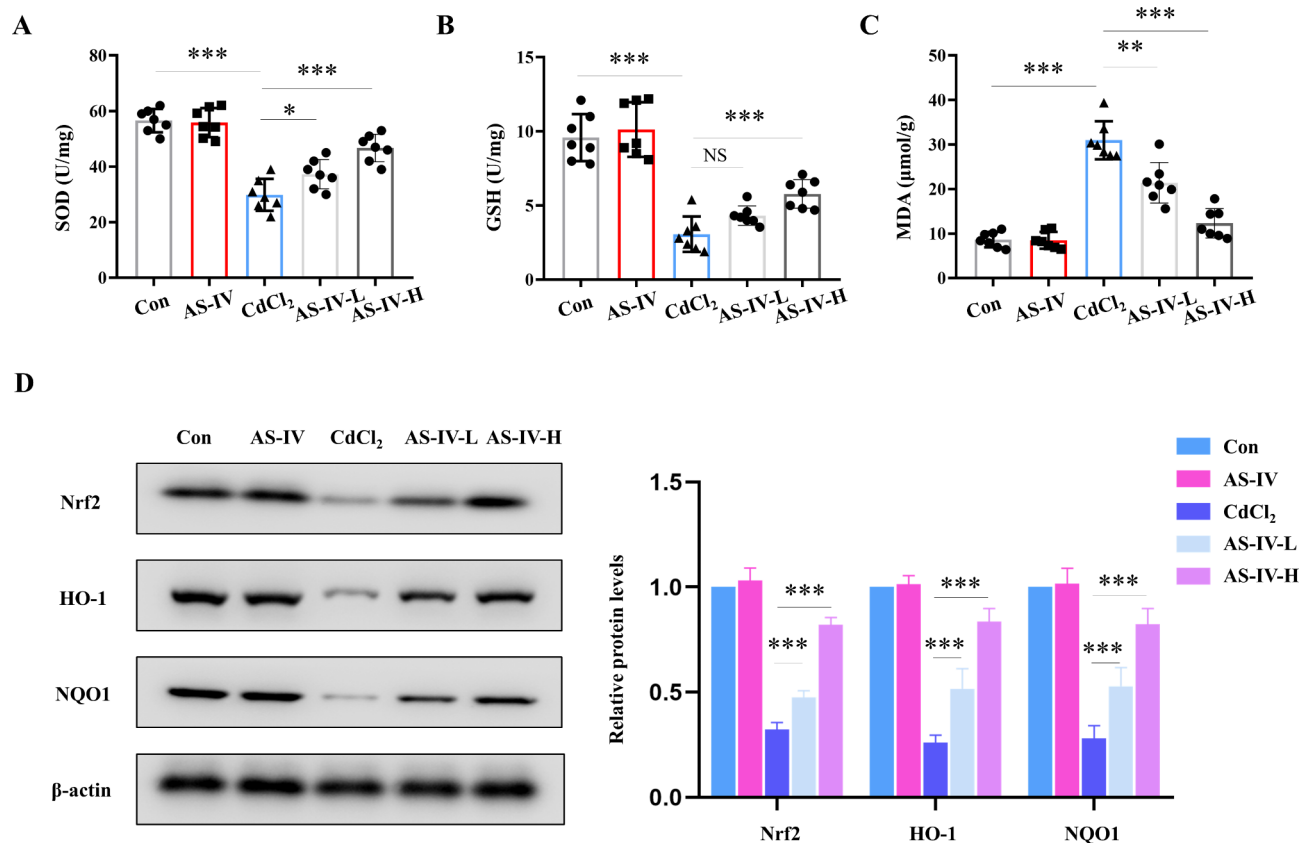
Fig. 5. *AS-IV* activation of Nrf2 inhibits cadmium-induced oxidative stress in the kidney. (**A**) Quantification of GSH levels using ELISA assay. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. $CdCl_2$, $p = 0.0395$ *AS-IV* -L vs. $CdCl_2$, $p < 0.001$ *AS-IV* -H vs. $CdCl_2$. (**B**) Quantification of MDA levels using ELISA assay. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. $CdCl_2$, $p = 0.0614$ *AS-IV* -L vs. $CdCl_2$, $p < 0.001$ *AS-IV* -H vs. $CdCl_2$. (**C**) Quantification of SOD levels using ELISA assay. Data are shown as mean ± s.d. $n = 7$. Two-tailed t-test. $p < 0.001$, Con vs. $CdCl_2$, $p = 0.00274$ *AS-IV* -L vs. $CdCl_2$, $p < 0.001$ *AS-IV* -H vs. $CdCl_2$. (**D**) Measurement of Nrf2, HO-1 and NQO1 protein expression by WB. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

and mitochondrial membrane potential alteration, contributing to apoptosis. In this study, we found that *AS-IV* could inhibit $CdCl_2$-induced ROS production and mitochondrial membrane potential alteration in HK2 cells through activation of the Nrf2 pathway and thus treat acute kidney injury. However, this study still has shortcomings. First, although the role of the Nrf2/HO-1 pathway in mediating the protective effects of AS-IV was emphasized in this paper, more in-depth mechanistic studies were lacking due to time and resource constraints. Secondly, heavy metal cadmium has long-term effects on nephrotoxicity, and this experiment also failed to verify whether the protective effect of AS-IV could be sustained for a longer period of time. Therefore, we will further address these issues in subsequent studies.

In recent years, more and more Chinese herbs have attracted the attention of researchers for their diverse and multi-targeted effects. Astragalus, as a commonly used Chinese herb, has been used clinically for more than 2,000 years. *AS-IV*, as a star component of Astragalus, is widely used in herbal preparations for the treatment of diabetes, such as Astragalus injection and Ginseng Radix Hypoglycemic Granules. However, what role *AS-IV* has in heavy metal cadmium-induced acute kidney injury is not clear, and this paper fills the gap in this field. It suggests that *AS-IV* may be a potential drug for the clinical treatment of heavy metal cadmium poisoning.

Conclusion
The present study demonstrated that *AS-IV* effectively alleviated oxidative stress and renal tubular injury induced by heavy metal cadmium and protected the kidneys of cadmium-intoxicated rats. In addition, *AS-IV* attenuated renal tubular cell injury by activating the NRF2 pathway to inhibit $CdCl_2$-induced ROS production and mitochondrial membrane potential alteration in HK2 cells. The results suggest that *AS-IV* has great potential as a viable therapeutic option as an antidote against cadmium toxicity.
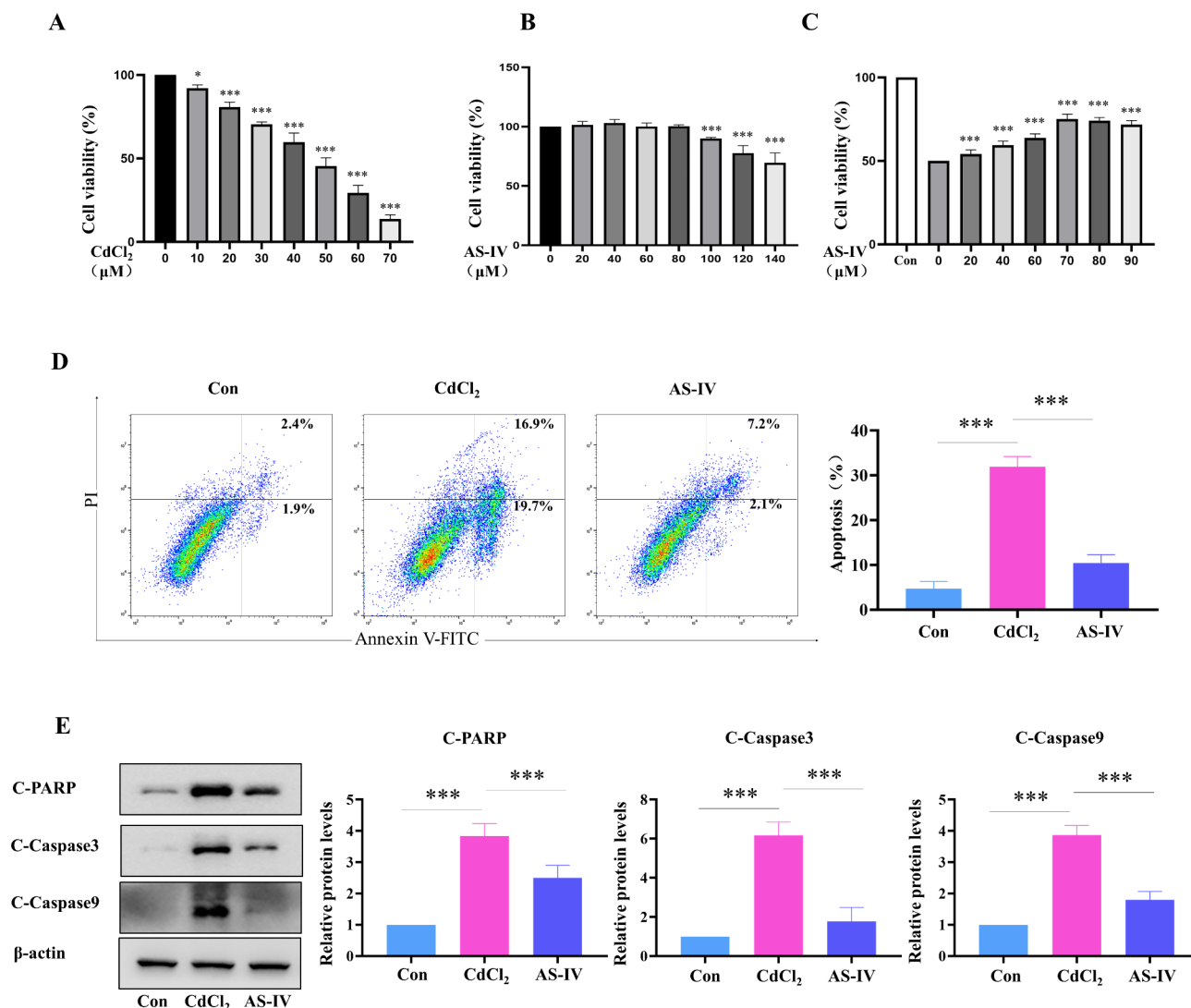
Fig. 6. *AS-IV* inhibits $CdCl_2$-induced apoptosis in HK2 cells. (**A**, **B**). HK-2 cells were incubated with different concentrations of $CdCl_2$ and *AS-IV* for 24 h respectively. Cell viability was assessed using the CCK-8 assay. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**C**) HK-2 cells were co-cultured with different concentrations of *AS-IV* with 50 μM $CdCl_2$ for 24 h and cell viability was assessed using the CCK-8 assay. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**D**) Apoptosis in HK2 cells was assessed by using flow cytometric assays. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**E**) Measurement of Cleaved-PARP, Cleaved-Caspase3 and Cleaved-Caspase9 protein expression by WB. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
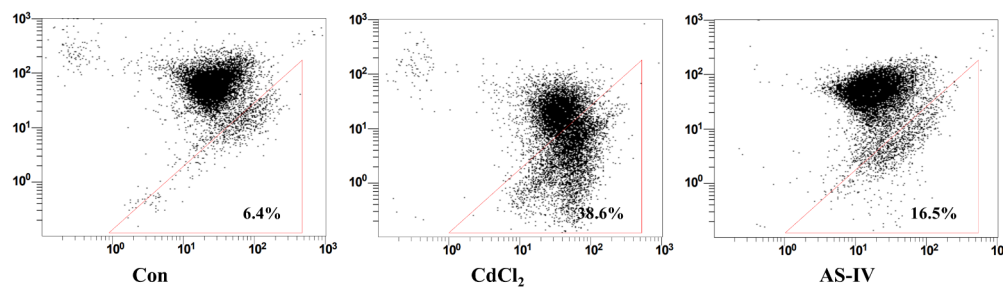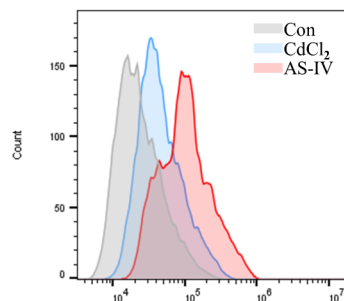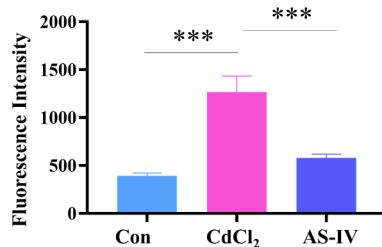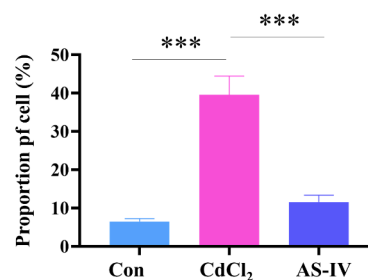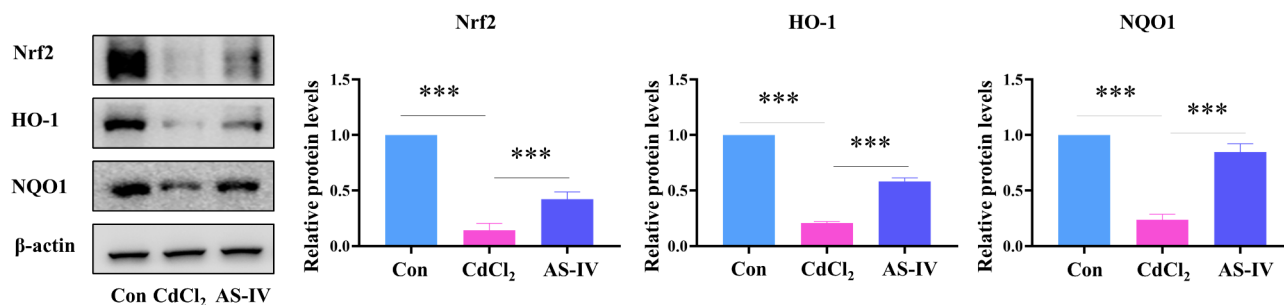
Fig. 7. *AS-IV* prevents cadmium-induced oxidative stress in HK2 cells by activating Nrf2 (**A**, **D**) Mitochondrial membrane potential alteration detected by JC-1 kit. (**B**, **C**) Intracellular ROS levels were detected by flow cytometry after staining with DCFH-DA probe. E. Measurement of HK2 cells Nrf2, HO-1 and NQO1 protein expression by WB. Data are shown as mean ± s.d. Two-tailed t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
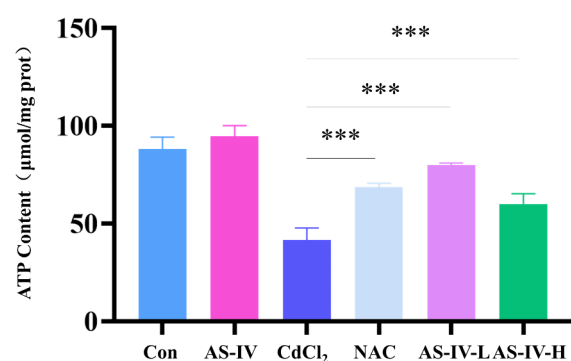
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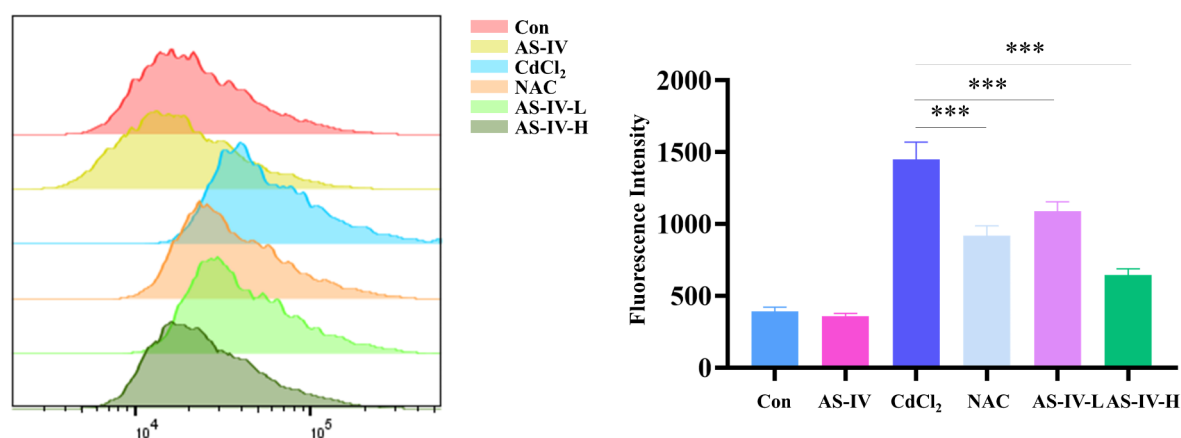
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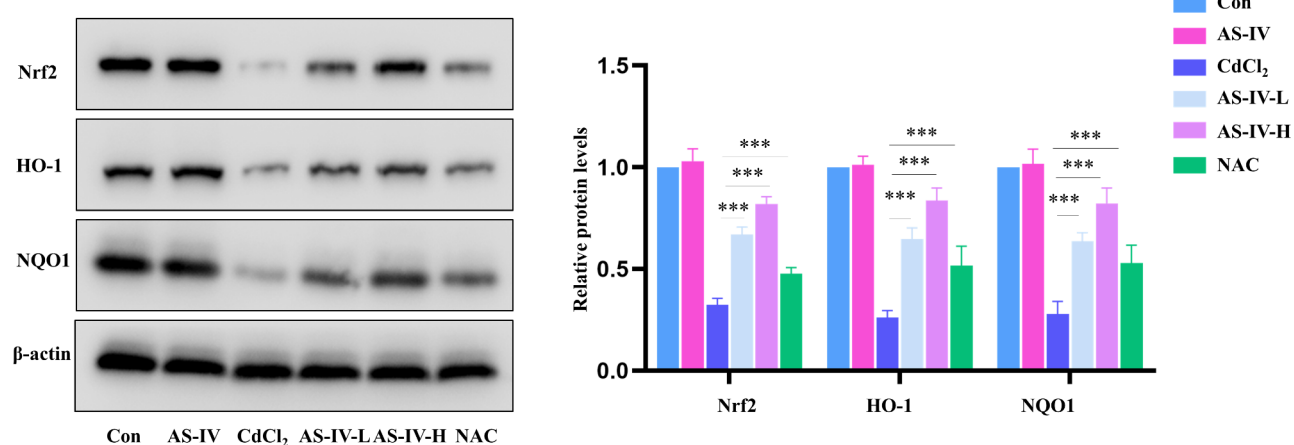
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Fig. 8. *AS-IV* has higher antioxidant capacity compared to NAC. (**A**) Intracellular ROS levels were detected by flow cytometry after staining with DCFH-DA probe. Data are shown as mean ± s.d. Two-tailed t-test. *** $p < 0.001$. (**B**) Measurement of Nrf2, HO-1 and NQO1 protein expression by WB. Data are shown as mean ± s.d. Two-tailed t-test. *** $p < 0.001$. (**C**) ATP content of HK2 cells. Measurement of Nrf2, HO-1 and NQO1 protein expression by WB. Data are shown as mean ± s.d. Two-tailed t-test. *** $p < 0.001$.

Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

YC.L and JY.Z, Methodology, performing the experiments, Formal analysis, Writing—original draft. TX.Z and XC.L perpared Figs. 1 and 2. C.W and ZY.Z perpared Figs. 3 and 4. JY.T perpared Figs. 5 and 6. XY.T, QY.H and WH.L Supervision.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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