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Original Article

Effect of astaxanthin on apoptosis of rat renal tubular epithelial cells induced by iohexol

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Abstract: Contrast acute kidney injury refers to acute renal failure due to the application of contrast agents. Astaxanthin, as an antioxidant, can improve early acute kidney injury in severely burned rats. However, the mechanism of astaxanthin for renal protection is still unclear. In this study, the rat renal tubular epithelial cells (NRK-52E) were treated with iohexol, astaxanthin, astaxanthin plus nicotinamide and nicotinamide. Subsequently, the nuclear morphology was observed by fluorescence staining of DAPI DNA, the apoptosis was detected by flow cytometry, the mitochondrial membrane potential was detected by JC-1 and the SIRT1, P53, Bax, Bcl-2 protein expression level was detected by Western blotting. We found that astaxanthin can reduce nuclear pyknosis and nuclear deep staining, decrease the number of apoptotic cells, up-regulate the expression of proapoptotic proteins P53 and Bax and up-regulate the expression of anti-apoptotic protein Bcl-2 by increasing SIRT1 expression level, thereby exerting protective effects on renal tubular epithelial cells. At the same time, nicotinamide has the opposite effect on the NRK-52E compared with astaxanthin. These results indicated that astaxanthin may provide a new option for the prevention of contrast-induced acute kidney injury.

Keywords: Astaxanthin, contrast-induced acute kidney injury, SIRT1, apoptosis

Introduction

With the development of coronary heart disease diagnosis, intervention and radiology diagnosis and treatment technology, the use of contrast agents is increasing. Contrast-induced acute kidney injury (CI-AKI) is gradually becoming one of the common causes of iatrogenic renal failure [1]. Simultaneously, CI-AKI has become the third major complication after PCI (followed after stent thrombosis and restenosis) [2]. The pathogenesis of CI-AKI is not fully understood, and a variety of factors might be involved in this pathophysiological process. Previous study indicates that oxygen free radical damage [3], direct tubular cytotoxic injury [4] and renal hemodynamic changes [5] were the important mechanisms of CI-AKI. At present, the research on the mechanism of CI-AKI and the protective drugs for CI-AKI are receiving more and more attention from clinicians. Some studies have found that astaxanthin, as a natural potent antioxidant, has been shown to have multiple cytoprotective effects such as anti-apoptosis [6]. In this study, we investigated the role of apoptosis during contrast-induced acute kidney injury in rats, and explored the anti-apoptosis protection of AST in this process.

Materials and methods

Main materials

Rat renal tubular epithelial cells (NRK-52E); Astaxanthin (sigma); Iohexol (Yangtze River Pharmaceutical Co., Ltd.); Fetal bovine serum (Sijiqing, China); DMEM/F12 medium (Hyclone, USA); Nicotinamide (Dalian Meilun Biotechnology Co., Ltd., China); BCA Protein Kit, Annexin V-FITC/PI Apoptosis Kit (Nanjing Kaiji Biotechnology Development Co., Ltd.); Mitochondria Membrane potential detection kit (JC-1) (Jiangsu Biyuntian Biotechnology Co., Ltd.); SIRT1 antibody (Absin Biotechnology Co., Ltd.); P53 antibody (Abclonal Biotechnology Co., Ltd.);

Bax antibody (Proteintech, USA); Bcl-2 Antibody (Santa Cruz, USA); β-actin antibody (Proteintech, USA).

Cell culture and grouping

NRK-52E cell line was cultured in DMEM/F12 medium containing 10% fetal bovine serum, 0.1% penicillin and streptomycin. When the cells grew to 70%~80% level, the cells were further cultured in DMEM/F12 medium without the serum for 24 h to be synchronized in the stationary growth period (G0/G1 phase). Then the cells were randomly divided into: blank control group (Control group), solvent control group (DMSO group), iohexol group (I group), astaxanthin pretreatment group (AST group), the astaxanthin and nicotinamide co-pretreatment group (AST+NA group), and the nicotinamide group (NA group).

Fluorescence staining of DAPI DNA to observe nuclear morphology

Rat renal tubular epithelial cells grown in 24-well culture plates were lightly washed 3 times (5 min each time) with PBS solution, fixed in 4% paraformaldehyde (dissolved in 0.1 mol/L PB, pH 7.4) at room temperature for 15 min, then the cells were gently washed again with PBS solution for 3 times (5 min each time), and add 3 ml of DAPI (4,6-diamidino-2-phenylindole, 4,6-diamino-2-phenylindole, fluorescent DNA binding dye, dissolved in PBS solution containing 0.2% Triton) for 5-10 min (This step needs to be protected from light), and washed with PBS solution for 3 times (5 min each time). After reaction, the cells were observed on the Olympus fluorescence microscope with excitation light at 400 nm and emitted light at 455 nm.

Annexin V-FITC/PI dual-labled flow cytometry to detect the apoptosis

Cells in the petri dish were gently rinsed with 2 mL of PBS solution and then remove the PBS solution. The cells were resuspended in the previous medium or pre-cooled 1 × Binding Buffer to a cell concentration of approximately 1×10^6 cells/ml. Take 500 μl of cell suspension (approximately 5×10^5 cells) into a clean centrifuge tube, centrifuge at 1000 g for 5 min, remove the supernatant, wash the cells once with 500 μl PBS solution, centrifuge, and remove the supernatant. Then 500 μl of 1 × Binding Buffer was added to wash the cells once, centrifuged, and the supernatant was removed. After resuspending the cells with 100 μl of 1 × Binding Buffer, 5 μL of Annexin V-APC was added to react for 15 min at room temperature without light. The cells were washed once with 500 μl of 1 × Binding Buffer, centrifuged, and the supernatant was removed. After resuspending the cells with 200 μl of 1 × Binding Buffer, 5 μl of PI was added. The samples were analyzed by flow cytometry 1 hour later.

Apoptosis mitochondrial membrane potential detection kit (JC-1) to detect the mitochondrial membrane potential

Discard the medium in the 6-well plate, wash it 3 times with PBS solution, add 1 ml of JC-1 staining working solution, mix thoroughly, incubate the cells in a cell incubator at 37°C for 20 min. During the incubation, an appropriate amount of JC-1 staining buffer (1 ×) was prepared by added distilled water to the JC-1 staining buffer (5 ×) in a ratio of four to one, and placed JC-1 staining buffer (1 ×) in an ice bath. After the incubation at 37°C, the supernatant was aspirated, washed twice with JC-1 staining buffer (1 ×), and 2 ml of the cell culture fluid was added, and observed under a fluorescence microscope. The excitation light can be set to 490 nm when the JC-1 monomer is detected, and the emitted light is set to 530 nm; when the JC-1 polymer is detected, the excitation light can be set to 525 nm, and the emitted light can be set to 590 nm.

Western blotting to analyze protein expression levels

After extracting total cellular protein, the protein concentration was determined by BCA, followed by polyacrylamide gel electrophoresis (SDS-PAGE), blocking at room temperature after transfection, then the protein was detected by immunoblot detection with specific antibodies, β-actin was used as an internal reference, chemiluminescence was used to colour development, and the target band was scanned for density. Repeat 3 times for each group.

Statistical analysis methods

The statistical experimental data, measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), Graphpad Prism 5.0 software was used to data analysis, and the difference

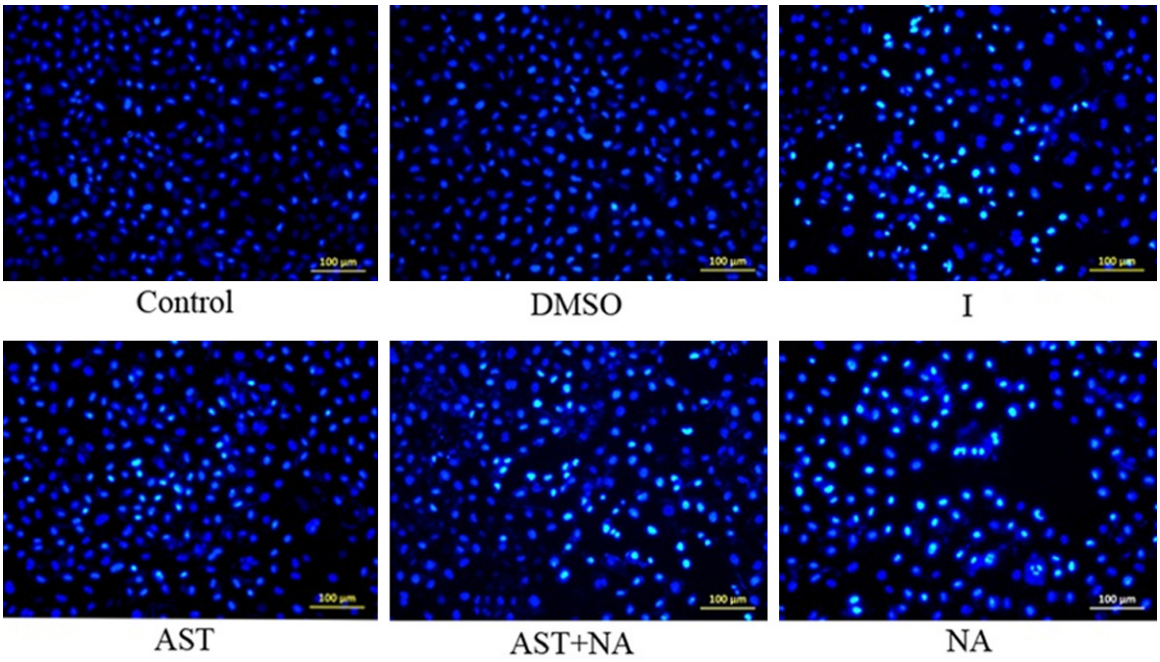

Figure 1. DAPI fluorescence staining of each group of NRK-52E cells (× 200).

between the groups was compared using variance analysis (one-Way ANOVA), q-test was used for comparison between groups, $P<0.05$ indicates that the difference was statistically significant.

Results

DAPI fluorescence staining was used to observe the nuclear morphology of each group

The morphology of nuclear of the epithelial cells was observed by DAPI DNA fluorescent staining (**Figure 1**). The control group and the DMSO group showed uniform nuclear staining and no apoptotic cells. The cells of I group were inferior to those of the control group. Some of the cells had highlighted nucleus pyknosis and nuclear deep staining, there were also some apoptotic cells with nuclear lysis. Compared with the I group, the AST group had less nuclear pyknosis and less nuclear deep staining, and decreased apoptotic cells. After administration of the SIRT1 inhibitor NA, the AST+NA group increased the number of condensed and brightened nucleus and increased apoptotic cells compared with the AST group. Compared with the AST+NA group, the cell damage in the NA group was further aggravated, and the number of apoptotic bodies was larger. The results shows that AST pretreatment can improve cell apoptosis, and NA can weaken the protective effect of AST on cells by blocking SIRT1 signaling pathway.

Annexin V-FITC/PI dual-labled flow cytometry to detect apoptosis rate

The apoptosis rate was detected by flow cytometry (**Figures 2** and **3**). Compared with the control group, the difference in the DMSO group was not statistically significant (P>0.05); compared with the control/DMSO group, the apoptosis rate in the I group was significantly increased ($^{a}P<0.05$); the apoptosis rate of AST group was significantly lower than that of I group ($^{b}P<0.05$), indicating that AST pretreatment can reduce the apoptosis of renal tubular epithelial cells induced by iohexol. After administration of the SIRT1 inhibitor NA, the apoptotic rate of the AST+NA group was significantly higher than that of the AST group ($^{c}P<0.05$). Compared with AST+NA group, the apoptosis rate in NA group was statistically significant ($^{d}P<0.05$); there was no significant difference in apoptosis rate between I group and AST+NA group (P>0.05), indicating that AST exerts anti-apoptotic effects mostly through the SIRT1 signaling pathway.

JC-1 to detect the mitochondrial membrane potential (MMP; Δψm)

Decreased mitochondrial membrane potential (Δψm) is an iconic event in the early stages of apoptosis. In this experiment, we used JC-1 staining to detect changes in Δψm. The relative

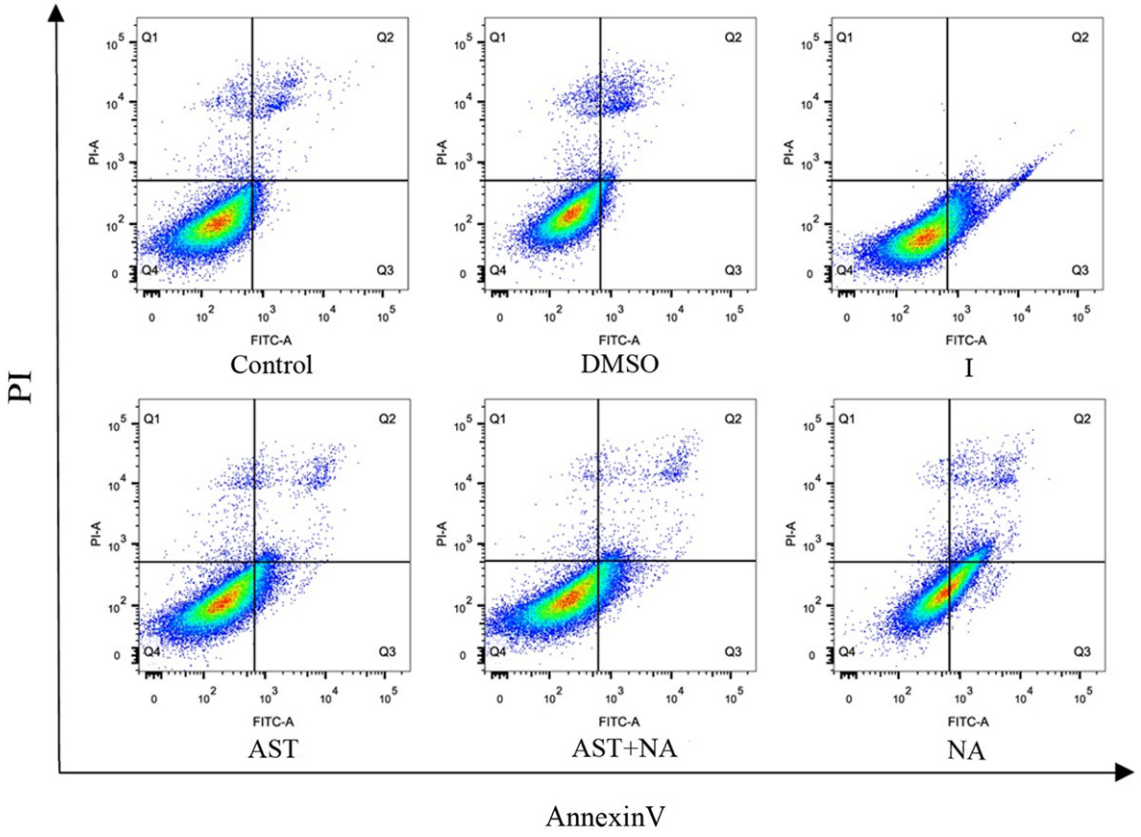

Figure 2. Apoptosis detected by flow cytometry after Annexin V/PI staining. Annexin V-/PI- represents living cells, Annexin V+/PI- represents early apoptotic cells, and Annexin V+/PI+ represents late apoptotic cells.

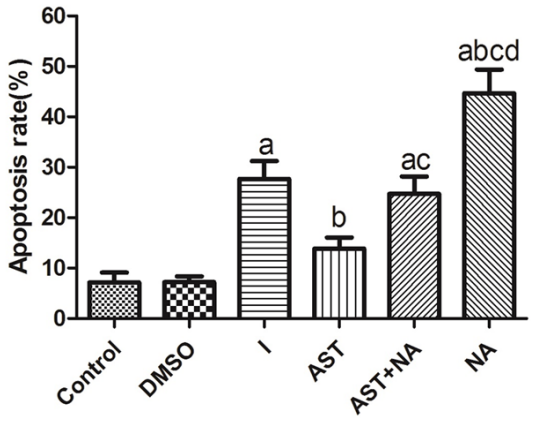

Figure 3. Apoptosis rate of NRK-52E cells in each group. [a]P<0.05, vs. control group alone; [b]P<0.05, vs. I group alone; [c]P<0.05, vs. AST group alone; [d]P<0.05, vs. AST+NA group alone.

proportion of red ang green fluorescence was usually used to measure the proportion of mitochondrial depolarization. The Δψm was detected by JC-1 (**Figure 4**). There was no significant difference between the control group and the DMSO group (P>0.05). The Δψm of the I group was significantly lower than that of the control group ([a]P<0.05). Δψm in the AST group was significantly higher than that in the I group ([b]P<0.05). Under the action of iohexol, there was no difference in Δψm between AST+NA group and I group (P>0.05). Compared with AST+NA group, AST group has significant growth in Δψm ([c]P<0.05), while Δψm in NA group decreased significantly ([d]P<0.05). The results suggest that AST can protect contrast agent induced renal tubular epithelial cell damage by improving the mitochondrial membrane potential pathway, and suggest that AST may play a role in the activation of SIRT1 signaling pathway.

Protein expression detected by Western blotting

Western-blot detection of SIRT1 protein expression levels in each group of cells: The SIRT1

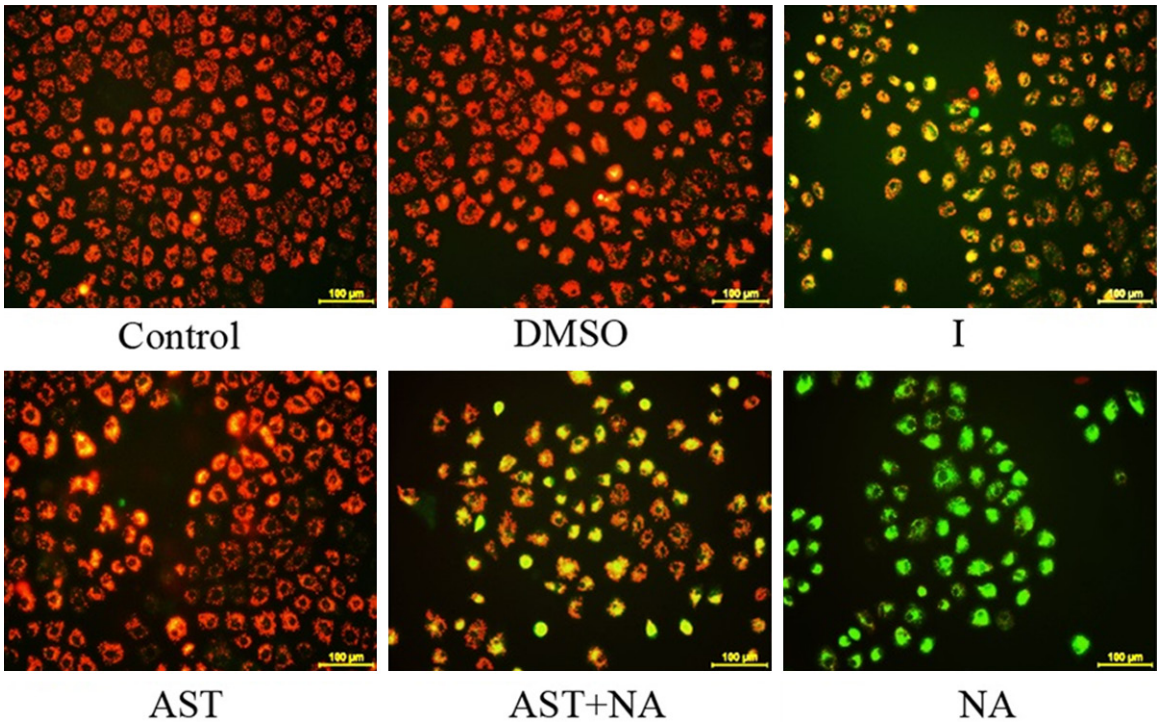

Figure 4. The mitochondrial membrane potential Δψm (× 200) detected by JC-1 fluorescent probe.

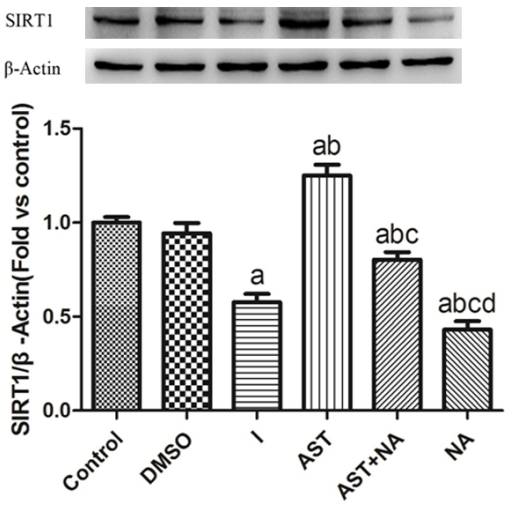

Figure 5. SIRT1 protein expression levels in NRK-52E cells by Western Blotting. [a]P<0.05, vs. control group alone; [b]P<0.05, vs. I group alone; [c]P<0.05, vs. AST group alone; [d]P<0.05, vs. AST+NA group alone.

protein expression was detected by Western-blot (**Figure 5**): There is no significant difference between the control group and DMSO group (P>0.05); compared with control/DMSO group, SIRT1 protein expression is decreased in I group ([a]P<0.05); SIRT1 protein expression in epithelial cells after AST pretreatment is significantly increased than the I group ([b]P<0.05); after administration of SIRT1 inhibitor NA, the content of SIRT1 protein in AST+NA group was significantly decreased compared with AST group ([c]P<0.05). The results indicate that AST pretreatment can up-regulate the expression of SIRT1 protein, while NA can reverse this effect of AST. Compared with the AST+NA group, the difference in the NA group was statistically significant ([d]P<0.05), which further suggesting the role of AST in activating the SIRT1 signaling pathway.

Western blotting detection of apoptosis-related protein (P53, Bax) expression levels and anti-apoptosis-related protein(Bcl-2) expression level in each group of cells: There was no significant difference between the control group and the DMSO group (P>0.05) (**Figure 6**). The expression of apoptosis-related proteins P53 and Bax were increased and the expression of anti-apoptosis-related protein Bcl-2 was decreased in I group compared with control group ([a]P<0.05); compared with I group, the expression of P53 and Bax protein decreased after AST pretreatment, while the expression of Bcl-2 protein increased ([b]P<0.05); after administration of SIRT1 inhibitor NA, compared with

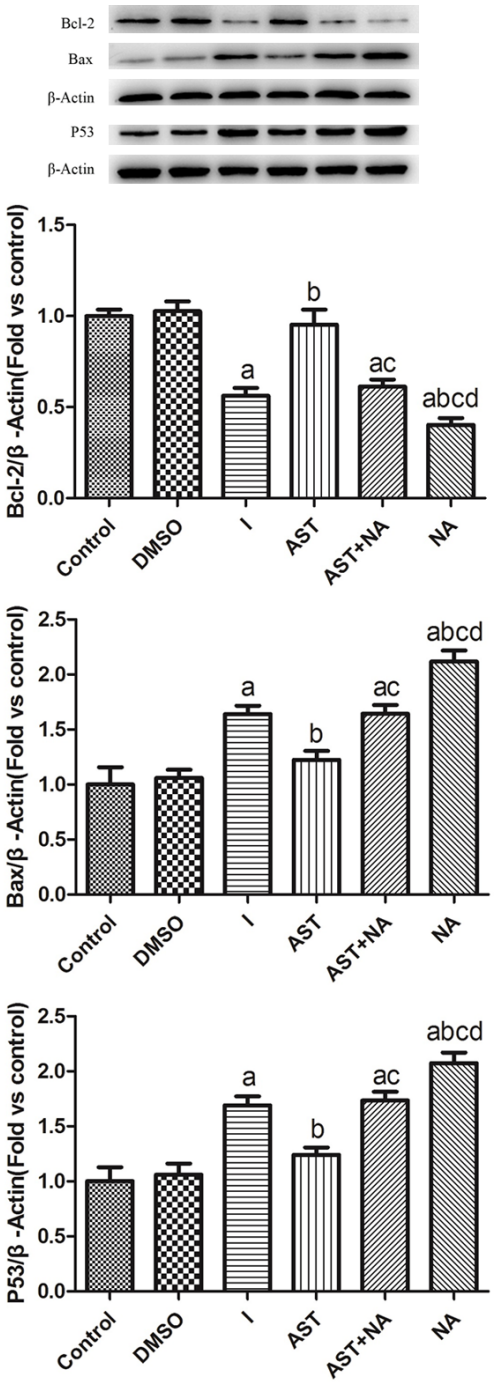

Figure 6. Expression levels of P53, Bax, Bcl-2 in NRK-52E cells in each group. [a]P<0.05, vs. control group alone; [b]P<0.05, vs. I group alone; [c]P<0.05, vs. AST group alone; [d]P<0.05, vs. AST+NA group alone.

AST group, the expression of P53 and Bax was significantly up-regulated, while the expression of Bcl-2 was significantly decreased ([c]P<0.05) in AST+NA group. Compared with the AST+NA group, the expression of P53 and Bax protein in the NA group was further increased, while the expression of Bcl-2 protein was more decreased ([d]P<0.05); and there was no statistical difference between the I group and the AST+NA group (P>0.05). These results indicate that AST pretreatment could regulate the expression of apoptosis-related proteins P53, Bax and anti-apoptosis-related protein Bcl-2 by activating SIRT1 signaling pathway, thereby improving contrast agent iohexol-induced apoptosis of renal tubular epithelial cells, while NA decreases SIRT1 protein expression to suppress the effect of AST.

Discussion

Contrast-induced acute kidney injury (CI-AKI) refers to the increase of serum creatinine (Scr) ≥44.2 μmmol/L (0.5 mg/dl) within 48~72 h after the application of contrast agent, or increased by ≥25% from baseline, and excluded from other causes of acute renal failure [7]. It is currently widely accepted that an effective measure to prevent CI-AKI is hydration therapy, which is a simple, effective and economical method to prevent of CI-AKI [8]. However, the use of elderly patients, hypertension or heart and renal insufficiency is limited. In addition, hydration therapy combined with drug intervention can further reduce the incidence of CI-AKI and improve the long-term prognosis of patients. Therefore, drug research has gradually become a research hotspot of CI-AKI prevention.

Astaxanthin (AST) is a natural red carotenoid derivative. It is widely found in many animals and plants, especially in marine organisms, such as microalgae, salmon, crabs and so on. Natural astaxanthin has various potentials, such as anti-oxidation, anti-inflammatory, immune regulation, inhibition of tumor growth, anti-aging, prevention of cardiovascular and cerebrovascular diseases, improvement of fertility, protection against ultraviolet rays, etc [9-12]. The research from Songxue Guo et al. [13] showed that astaxanthin can improve early acute kidney injury in severely burned rats by reducing the apoptosis of renal tubular cells through reducing the oxidative stress induced by burn and regulating the mitochondrial pro-apoptotic protein pathway. Consistent with this study, our experimental results showed that contrast agent can induce apoptosis of rat renal tubular epithelial cells, and pretreating

the cells with AST can significantly reduce contrast-induced apoptosis. Specifically, compared with control group, nuclear condensation and nuclear deep staining were observed and apoptotic bodies appeared; Δψm decreased significantly, and apoptosis rate increased significantly in I group; while AST group had improved nuclear condensation and deep nuclear staining state, and less apoptotic cells, Δψm increased, and the apoptosis rate decreased significantly compared with I group.

Apoptosis refers to an autonomic, programmed death process which is regulated by gene after a cell receives a stimulus signal [14-16]. There are mainly three signaling pathways, among which the mitochondrial pathway plays a leading role, and it is closely related to Cyt c, AIF, ROS, Bcl-2, P53 and other molecules [17-20]. The Bcl-2 family is the key to the mitochondrial apoptotic pathway which plays an important role in the process of apoptosis. It can be divided into two categories: one is the anti-apoptotic gene represented by Bcl-2; the other is the pro-apoptotic gene represented by Bax. They regulate mitochondrial membrane permeability and integrity by activating a cascade of downstream genes, thereby regulating the release of mitochondrial pro-apoptotic factor and participating in mitochondria-mediated apoptosis [21]. Some studies have found that P53 regulates apoptosis by regulating the Bcl-2 family [22]. The P53 gene is an important tumor suppressor gene and an important apoptosis-related gene. The target gene of p53 encodes a variety of pro-apoptotic proteins, such as PIGs, Fas, Apaf-1, Bax, PUMA, etc. which means P53 can act as a transcription factor to regulate the expression of downstream apoptosis-related proteins and promote apoptosis [23]. It has been reported that P53 also could directly binds to and activates Bak, induces Bak oligomerization, thereby inducing Cytc release and apoptosis [24, 25]. In addition, some scholars believe that p53 can also directly stimulate mitochondria to release ROS to induce apoptosis [26].

Silencing Regulatory Factor 2 Associated Enzyme 1 (SIRT1) is a NAD+ dependent class III histone deacetylation enzyme which was found in mammals and it has the highest homology to the yeast chromatin silencer (silent information regulator 2, Sir2). The downstream effector molecules of SIRT1 include histone, P53, NF-κB, peroxisome proliferator-activated receptor (PPARγ) and helper activators (PGC-1α), FOXOs, HIF1, etc., which regulate glycolipid metabolism, inhibition of oxidative stress and inflammation, reduction of apoptosis, reduction of aging and other biological effects by deacetylating them, thereby playing an important role in anti-aging, tumor, glycolipid metabolism homeostasis and cardiovascular system [27-30]. Recently, SIRTI has been continuously researched in the kidney field. Studies have shown that SIRTI can improve a variety of acute and chronic kidney diseases, delay the process of kidney aging, and improve the prognosis of kidney disease.

In this experiment, astaxanthin can inhibit the CI-AKI by improving oxidative stress and apoptosis through the mitochondrial pathway, and SIRT1 can achieve antioxidant and anti-apoptotic effects by acting on downstream effector molecules, so we conjecture that astaxanthin works by activating the SIRT1 signal. We found that SIRT1 expression decreased after application of iohexol, and SIRT1 expression was significantly higher than that of group I after astaxanthin intervention. To further confirmation, we used the specific inhibitor of SIRT1, Nicotinamide (NA), to inhibit the function of SIRT1. The results showed that the content of SIRT1 protein in NRK-52E cells was significantly decreased after NA administration, indicating that SIRT1 was successfully inhibited. At the same time, we also found that compared with the AST group, the expression of pro-apoptotic proteins P53 and Bax in AST+NA group increased, and the expression of anti-apoptotic protein Bcl-2 decreased. The results indicated that astaxanthin protects NRK-52E cells by activating SIRT1 signal and the SIRT1 acts upstream of these molecules. In addition, compared with the AST+NA group, the NA group lacked the protection of AST. The expression of pro-apoptotic proteins P53 and Bax were further up-regulated in the NA group, while the anti-apoptotic protein Bcl-2 was further down-regulated.

Collectively, these results reveal that astaxanthin can improve contrast-induced acute kidney injury by stabilizing mitochondrial membrane potential, activating SIRT1 pathway, down-regulating pro-apoptotic proteins P53, Bax, and up-

regulating anti-apoptotic protein Bcl-2. Astaxanthin provides a new choice for the prevention and treatment of CI-AKI, but the specific mechanism of the protective effect of astaxanthin on the kidney has not been fully elucidated, and further research is needed.

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Disclosure of conflict of interest

None.

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