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ORIGINAL ARTICLE

Astaxanthin Attenuates Contrast-Induced Acute Kidney Injury through Silent Mating-Type Information Regulation 2 Homolog-1/Peroxisome Proliferator-Activated Receptor γ Co-activator–α/NRF1 Signaling Pathway

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ABSTRACT

Objectives: In this study, we established a model of contrast-induced acute kidney injury with iohexol. This model was used to investigate whether astaxanthin (AST) attenuates oxidative stress and apoptosis by activating silent mating-type information regulation 2 homolog-1 (SIRT1) signaling pathway in rat renal tubular epithelial cells (NRK-52E). **Materials and Methods:** NRK-52E cells were randomly divided into six groups: control group (CON group), vehicle (dimethyl sulfoxide group, iohexol group (I group), AST pretreatment group (AST + I group), AST plus nicotinamide (NA) co-pretreatment group (AST + NA + I group), and NA treatment group (NA + I group). The cellular activity was measured by cell counting kit-8. We estimated the levels of malonaldehyde (MDA) by the thiobarbituric acid method, and the level of intracellular reactive oxygen species (ROS) was measured by flow cytometry. Western blot analysis was conducted to detect the protein levels of SIRT1, peroxisome proliferator-activated receptor γ co-activator–α (PGC-1α), and NRF1. **Results:** Compared with the CON group, the I group showed suppressed cellular activity; increased levels of MDA and ROS; and decreased levels of SIRT1, PGC-1α, and NRF1 protein. Compared with the I group, AST + I pretreated group showed high cellular activity; low levels of MDA and ROS; and increased levels of SIRT1, PGC-1α, and NRF1 protein. SIRT1 inhibitor NA reversed the protective effect of AST. Compared with the AST + NA + I group, NA + I group showed low cellular activity; high levels of MDA and ROS; and low levels of SIRT1, PGC-1α, and NRF1 protein, which further confirmed the protective effect of AST. **Conclusion:** AST alleviated iohexol-induced NRK-52E cell injury by decreasing the formation of MDA and ROS and upregulating the SIRT1/PGC-1α/NRF1 signaling pathway.

Key words: Astaxanthin, contrast-induced acute kidney injury, oxidative stress, peroxisome proliferator-activated receptor γ co-activator–α, silent mating-type information regulation 2 homolog-1

SUMMARY

- Astaxanthin (AST), is a strong antioxidant, has a protective effect on contrast-induced acute kidney injury (CI-AKI), but its mechanism is still unknown. In this study, the rat renal tubular epithelial cells (NRK-52E) were treated separately with iohexol, AST, AST + nicotinamide (NA), and NA. We found that AST can reduce the number of apoptotic cells, downregulate the level of malonaldehyde and reactive oxygen species, and upregulate the expression of proliferator-activated receptor γ co-activator–α and NRF1 by increasing the expression level of silent mating-type information regulation 2 homolog-1, thereby exerting protective effects. Meanwhile, NA has the opposite effect in NRK-52E cells compared with astaxanthin. These results demonstrate that astaxanthin might be a new option for the prevention of CI-AKI.

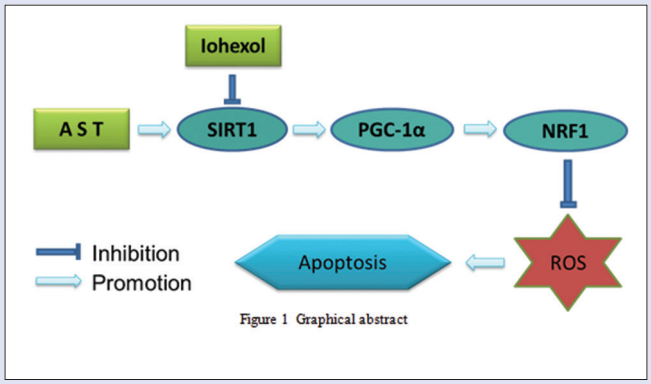

Figure 1 Graphical abstract

Abbreviations used: AST: Astaxanthin; NRK-52E: Rat renal tubular epithelial cells; I: Iohexol; DMSO: Dimethyl sulfoxide; MDA: Malonaldehyde; ROS: Reactive oxygen species; CI-AKI: Contrast-induced acute kidney injury; SIRT1: Silent mating type information regulation 2 homolog-1; PGC-1α: Peroxisome proliferator - activated receptor γ co-activator – α; NRF1: Nuclear respiratory factor 1; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS: Phosphate balanced solution

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INTRODUCTION

Along with the widespread application of contrast agents in cardiac interventional surgery, contrast-induced acute kidney injury (CI-AKI) has gradually drawn the attention of cardiologists.[1] CI-AKI refers to the acute renal impairment that occurs after the intravascular administration of iodine contrast agents, excluding other reasons that can cause acute renal failure.[2] Although the exact pathophysiological mechanism of

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CI-AKI is not yet clear,[3] it has been hypothesized that oxidative stress injury is one of the primary pathogenesis.[4] Astaxanthin (AST) is a red carotenoid derivative obtained from various natural sources.[5] Previous studies have shown that AST can reduce the apoptosis of the renal tubular cells of rats induced by contrast agents, downregulate the expression of proapoptotic proteins p53 and Bax.[6] Therefore, in this study, we aimed to investigate whether AST alleviates renal epithelial cells injury induced by iohexol. We further explored the changes in the protein expression of silent information regulator 2 homolog-1 (SIRT1), peroxisome proliferator-activated receptor γ co-activator–α (PGC-1α), and nuclear respiratory factor 1 (NRF1). This study provides a theoretical basis for the clinical treatment of CI-AKI.

MATERIALS AND METHODS

Cells and chemicals

The following chemicals were obtained in this study: rat renal tubular epithelial cells (NRK-52E), AST (sigma), iohexol (Yangtze River Pharmaceutical Industry Co., Ltd), fetal bovine serum (FBS, Evergreen, China), Dulbecco's modified Eagle medium (DMEM)/F12 (Hyclone, USA), nicotinamide (NA) (Dalian Meilun Biotechnology Co., Ltd., China), BCA protein kit (Beyotime Biotechnology Company), cell counting kit (CCK)-8 kit (Dojindo Laboratories, Kumamoto, Japan), reactive oxygen species (ROS) assay kit and malondialdehyde (MDA) (Beyotime Biotechnology Company), β-actin antibody (Proteintech, USA), SIRT1 antibody (Absin Biotechnology Co., Ltd), PGC-1α antibody (Proteintech, USA), and NRF1 antibody (Proteintech, USA).

Cell culture and study groups

NRK-52E cells were purchased from the pharmacology laboratory of Xuzhou Medical University and were cultured in an incubator with a constant temperature (5% CO_2, 37°C) in the DMEM/F12 medium containing of 10% FBS and 0.1% penicillin and streptomycin. The medium was changed every 2 days. NRK-52E cells were randomly divided into six groups: control (CON) group, vehicle (dimethyl sulfoxide [DMSO]) group (0.1% DMSO), I group (50 g/L iohexol), AST + I group (50 g/L I + 20 μmol/L AST), AST + NA + I group (50 g/L iohexol + 20 μmol/L AST + 20 mmol/L NA), and NA + I group (50 g/L iohexol + 20 mmol/L NA).

Determination of cell proliferation with cell counting kit-8 kit

After digestion with trypsin, the cell suspension was centrifuged and the cell pellet was diluted with DMEM/F12 medium containing 10% FBS to obtain a cell density of 5×10^4/mL. From this, 100 μL/well (5000 cells) were inoculated into 96-well plates, and the cells were cultured in the carbon dioxide incubator (5% CO_2) (Steri-Cycle 371, Thermo Scientific, USA) at 37°C and 100% relative humidity for 24 h. The same volume of cell- and drug-free medium was added to the negative CON group. Then, 48 h after the intervention, 100 μL medium containing 10 μL CCK-8 solution was added to each well, and the cells were incubated for 2 h in an incubator with a constant temperature (5% CO_2, 37°C, 100% relative humidity). The optical density was read at 450 nm using a microplate reader.

Measurement of the intracellular reactive oxygen species levels by flow cytometry

After digestion, centrifugation, and counting, the cells were inoculated into 6-well plates at a density of 4×10^5/mL and cultured for 24 h. Then, based on their group, the cells were treated with corresponding drugs. Negative control cells did not receive any treatment. Rosoup (1 μL) + dichlorodihydrofluorescein diacetate (DCFH-DA) (1 mL) was added to the positive control group. After this, 1 mL of fluorescent probe DCFH-DA was added at a final concentration of 10 mol/L to each group except for the negative control group. Then, the cells were incubated for 30 min in an incubator (5% CO_2, 37°C, 100% relative humidity). Thereafter, the medium was discarded and the cells were washed thrice in a serum-free medium (5 min/wash) to completely remove the extracellular dye. After digestion with trypsin, the cells were centrifuged and were read on the flow cytometer (FACSCantoII, Becton-Dickinson/BD, USA) with the following parameters: excitation and emission wavelengths of 488 nm and 525 nm, respectively, and then, the ROS level was detected. The above steps were repeated thrice.

Determination of malonaldehyde

MDA was detected using MDA assay kit (Beyotime Biotechnology Company). The thiobarbituric acid method was used to measure the MDA content in the cell supernatant. After lysis, the cells were centrifuged at 1600 *g* for 10 min, and the supernatant was collected for subsequent measurement. Briefly, 100 μL sample was added to the centrifuge tube; lysis solution and standard were used as negative and positive control, respectively. Then, 200 μL MDA detection solution was added (prepared according to the kit instructions). After mixing, the solution was heated at 100°C for 15 min and then cooled naturally. After cooling, the solution was centrifuged at 1000 *g* for 10 min at room temperature. Then, 200 μL supernatant was added to a 96-well plate, and then the OD was read at 532 nm using the microplate reader. All experiments were repeated thrice.

Western blot analysis

The total concentration of the extracted protein was measured by the BCA kit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted to isolate proteins. The separated proteins were transferred onto PVDF membranes and blocked at room temperature. The primary antibodies (β-actin [1:2000], SIRT1 [1:500], PGC-1α [1:1000], and NRF1 [1:500]) were added and incubated at 4°C overnight. The secondary antibody was incubated at room temperature for 2 h. Chemiluminescence reagents were used to develop the color, and the results were scanned. Finally, scanning density analysis was conducted for the target bands. Gray value analysis was conducted with Image J analysis software with β-actin as the internal reference. All experiments were repeated thrice.

Statistical analysis

All experimental data are presented as mean ± standard deviation. GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, California, USA) was used to analyze the data. The differences in the mean between the groups were compared using one-way analysis of variance. Then, *q*-test was used to compare the data between groups. $P < 0.05$ indicates that the difference was statistically significant.

RESULTS

Comparison of cell proliferation activity

According to the CCK-8 analysis, there was no significant difference between the DMSO group and the CON group ($P > 0.05$). The proliferation of cells in the I group decreased compared with the CON group ($P < 0.05$). The proliferation of NRK-52E cells in the AST group was more compared to that of the I group ($P < 0.05$). After the administration of the SIRT1 inhibitor (NA), the proliferation of NRK-52E cells in the AST + NA group was significantly decreased compared to the AST group ($P < 0.05$). The difference between the AST + NA group and the NA

group was statistically significant ($P < 0.05$), which further confirmed the protective effect of AST. Our results showed that pretreatment with AST might improve cell proliferation and NA might weaken the protective effects of AST by blocking the SIRT1 signaling pathway [Figure 1].

Comparison of malonaldehyde content in NRK-52E cells

When there is a damage to the cell or the organism, ROS production increases. Excessive production of ROS initiates lipid peroxidation process with the side chains of polyunsaturated fatty acids associated with phospholipids, enzymes, and membrane receptors. Lipid peroxidation forms peroxides such as MDA and 4-hydroxynonenal. In this study, we did not obtain any statistically significant differences between the DMSO group and the CON group ($P > 0.05$). Compared with the CON group, the MDA level in the I group was increased ($P < 0.05$), whereas in the AST + I group, it was significantly decreased ($P < 0.05$). After the administration of SIRT1 inhibitor (NA), the MDA level in the AST + NA + I group was significantly higher than that of the AST + I group ($P < 0.05$). According to our results, the decrease in the levels of MDA by AST can be significantly reversed by NA, which further verified that AST played a protective role through the SIRT1 signaling pathway [Table 1].

Comparison of the reactive oxygen species level in NRK-52E cells

The average fluorescence intensity was analyzed with flow cytometry to analyze the intracellular ROS level. Compared with the CON group, there was no significant change in the DMSO group with regard to the intracellular ROS level ($P > 0.05$), whereas in the I group, it was significantly increased ($P < 0.05$). After the administration of AST, intracellular ROS activity was significantly reduced compared with the I group ($P < 0.05$). After the administration of NA, the ROS level increased in the AST + I group ($P < 0.05$). The difference between the AST + NA + I group and the NA + I group was statistically significant ($P < 0.05$), which further confirmed the protective effect of AST. According to our results, AST reduced the intracellular ROS level, whereas NA significantly reversed the decrease in the production of ROS [Table 1 and Figure 2]. These results were consistent with the intracellular MDA level.

Table 1: Malonaldehyde and reactive oxygen species levels of NRK-52E cells in each group ($\bar{x}\pm s$, $n=3$)

Groups	MDA (nmol/mg)	Mean ROS fluorescence intensity
CON	3.21±0.80	366.33±81.86
DMSO	3.91±1.38	440.67±74.78
I	11.23±1.89[a]	862.00±135.71[a]
AST + I	6.99±1.15[ab]	567.67±114.67[b]
AST + NA + I	10.84±0.81[ac]	800.00±164.61[ac]
NA + I	17.42±2.19[abcd]	1517.33±163.66[abcd]

[a]$P<0.05$, compared with CON group; [b]$P<0.05$, compared with I group; [c]$P<0.05$, compared with AST + I group; [d]$P<0.05$, compared with AST + NA + I. MDA: Malonaldehyde; ROS: Reactive oxygen species; CON: Control; DMSO: Dimethyl sulfoxide; AST: Astaxanthin; NA: Nicotinamide; I: Iohexol

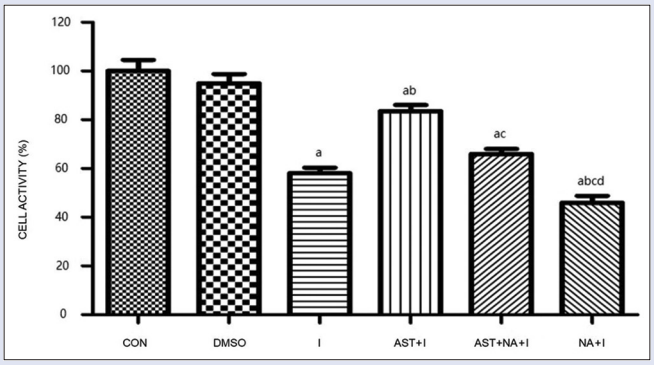

Figure 1: The cell activity of NRK-52E cells in each group was tested. [a]$P < 0.05$, compared with CON group; [b]$P < 0.05$, compared with I group; [c]$P < 0.05$, compared with AST group; [d]$P < 0.05$, compared with AST + NA. Con: Control; AST: Astaxanthin; NA: Nicotinamide

Western blot analysis

According to the results of the Western blot analysis, there was no significant difference between the protein expression of SIRT1, PGC-1α, and NRF1 in the DMSO group and the CON group ($P > 0.05$), whereas it decreased in the I group ($P < 0.05$). Compared with the I group, the protein levels of SIRT1, PGC-1α, and NRF1 in the AST + I pretreated group were significantly higher ($P < 0.05$), which indicated that AST pretreatment can regulate the expression of oxidative stress-related proteins PGC-1α and NRF1 by activating SIRT1 protein expression and improve the oxidative stress injury of renal tubular epithelial cells induced by iohexol. After the administration of NA, the protein expression of SIRT1, PGC-1α, and NRF1 by NRK-52E cells was significantly reduced compared with the AST + I group ($P < 0.05$). Compared with the AST + NA + I group, the protein expression of SIRT1, PGC-1α, and NRF1 in the NA + I group was found to be reduced, and the difference was statistically significant ($P < 0.05$). Furthermore, we found that AST regulated the expression of the oxidative stress-related proteins (PGC-1α and NRF1) through the SIRT1 signaling pathway and that the protective effect of AST could be significantly reversed after the blockage of SIRT1 by NA [Figure 3].

DISCUSSION

AST is a carotenoid-containing ketone group, which shows strong antioxidant capacity due to its long conjugated unsaturated double bond structure.[7] AST is a natural compound with multiple potentials.[8,9] Previous studies have shown that the AST pretreatment significantly reduced oxidative stress and inflammation in the kidney samples of I/R mice.[10] Consistent with the aforementioned studies, we discovered that iohexol can significantly inhibit the cell viability of NRK-52E cells, with significantly increased level of MDA and ROS compared with the CON group. Furthermore, the 24-h AST pretreatment could significantly improve the cell viability and reduce the intracellular levels of MDA and ROS.

Currently, the exact pathophysiological mechanism of CI-AKI has not been fully understood. Majority of the researchers believe that oxidative stress injury is an important pathogenesis. Previous studies on animals have found that[11,12] after the use of the contrast agent, the level of MDA and LPO increases, whereas the levels of SOD, GSH, and peroxide kinase decrease. After treatment with antioxidants, the level of MDA and LPO decreased and the levels of SOD and GSH increased. The results of this study are consistent with previous conclusions. We found that ROS levels decreased by 34.15% and MDA levels decreased by 37.76% in the AST group compared with the I group. Pflueger *et al.*[13]also verified that one of the causes of acute renal injury is increase in the level of ROS or decrease in the antioxidant enzyme activity.

Oxidative stress refers to the accumulation of ROS and reactive nitrogen species which is stimulated by harmful substances, thereby resulting in cellular damage.[14] Mitochondria play an important role in the oxidative stress. When mitochondria are dysfunctional, the level of ROS increases, and excessive levels of ROS would directly attack mitochondria, thereby

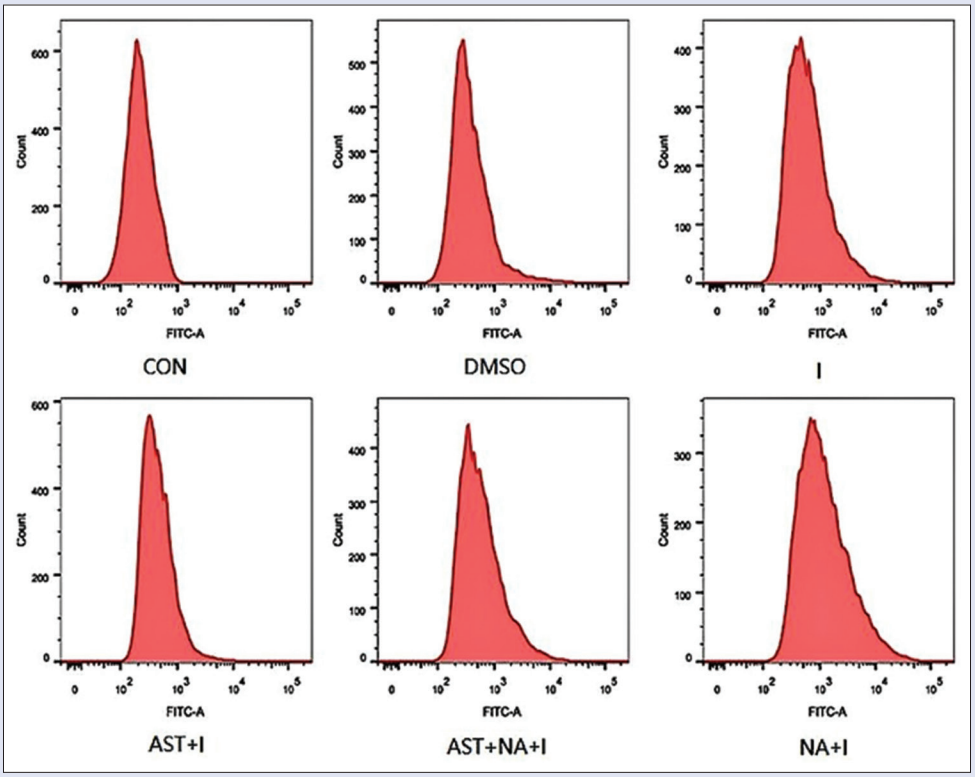

Figure 2: The reactive oxygen species levels of NRK-52E cells in each group were tested

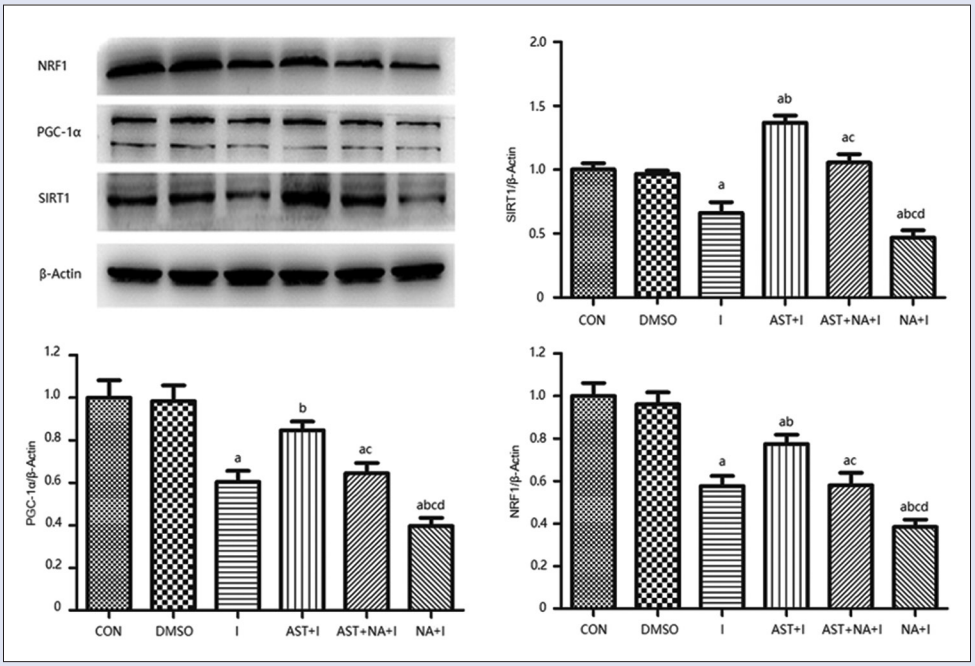

Figure 3: Protein expression levels of silent mating-type information regulation 2 homolog-1, peroxisome proliferator-activated receptor γ co-activator–α, and NRF1 in each group. [a]$P < 0.05$, compared with CON group; [b]$P < 0.05$, compared with I group; [c]$P < 0.05$, compared with AST group; [d]$P < 0.05$, compared with AST + NA. AST: Astaxanthin; NA: Nicotinamide

resulting in decreased mitochondrial membrane permeability, decreased ATP synthesis, and increased ROS production. These steps occur one after the other.[15] PGC-1α, a transcriptional co-activator of PPARγ, plays a significant role in the oxidative metabolism and mitochondrial biosynthesis.[16,17] The expression of PGC-1α is tissue-specific and is highly expressed in organs rich in mitochondria or with high energy requirements, such as the heart, liver, kidney, brain, skeletal muscle, and brown fat. Many studies have verified that PGC-1α plays a key role

in the process of mitochondrial biosynthesis. When combined with its downstream target gene NRF1, PGC-1α can transcriptionally activate the expression of Tfam, TFBlM, and TFB2M, which are the target genes of NRF1. Thus, PGC-1α can participate in the transcriptional regulation of mitochondrial biosynthesis.[18]

SIRT1, a NAD-dependent histone deacetylase of class III,[19] regulates cell metabolism, slows down aging, and inhibits oxidative stress and reduces apoptosis through deacetylation of the downstream target molecules such as P53, FOXOs, PGC-1α, HIF2, and Atg5, SIRT1.[20,21] In a previous study conducted on I/R kidney injury in rats,[22,23] it was found that SIRT1 protected the kidneys mainly by the deacetylation of PGC-1α and maintaining the membrane stability of mitochondria under stress. In this study, the expression levels of SIRT1, PGC-1α, and NRF1 protein in NRK-52E cells in the I group were significantly lower than that in the CON group, and the expression levels of SIRT1, PGC-1α, and NRF1 were increased after the AST pretreatment. Furthermore, the levels of SIRT1 protein were significantly reduced and the expressions of PGC-1α and NRF1 were also reduced after NA treatment. According to our results, AST protects NRK-52E cells by activating the SIRT1 signaling pathway, whereas SIRT1 plays an important role in the upstream of these molecules. In addition, compared with the AST + NA + I group, the NA + I group had decreased cellular activity, increased MDA and ROS levels, increased protein levels of SIRT1, PGC-1α, and NRF1 due to the lack of protective effect of AST.

CONCLUSION

In summary, the results of this study demonstrate that the increased levels of ROS and MDA and the decreased level of SIRT1, PGC-1α, and NRF1 protein promoted the oxidative stress injury in NRK-52E cells. AST can alleviate the condition of CI-AKI, whose main mechanism is to activate the SIRT1 signaling pathway, upregulate the expression of PGC-1α and NRF1, and reduce mitochondria-related oxidative stress, which provides a new choice for the prevention of CI-AKI.

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Conflicts of interest

There are no conflicts of interest.

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