

SDRP Journal of Food Science & Technology (ISSN: 2472-6419)

Astaxanthin attenuate iohexol-induced human proximal renal tubular epithelial cells injury via the ROS/NLRP3 inflammasome signal pathway

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DOI: 10.25177/JFST.4.3.RA.503

Research

Received Date: 15th Apr 2019Accepted Date: 06th May 2019Published Date: 10th May 2019

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CITATION

Dongmei Gao, Shun Yao, Yanhu Sun, Di Zheng, Quan Zhang, Wenhua Li, Astaxanthin attenuate iohexol-induced human proximal renal tubular epithelial cells injury via the ROS/NLRP3 inflammasome signal pathway (2019) SDRP Journal of Food Science & Technology 4(3)

ABSTRACT

BACKGROUND: To investigate the possible mechanism of renal protection of astaxanthin in iohexol-induced human proximal renal tubular epithelial cells injury.**METHODS:** Human proximal renal tubular epithelial cells (HK-2) were randomly divided into six groups: blank control group (Control group); Dimethyl sulfoxide solvent control group (DMSO group); astaxanthin control group (AST group); Contrast media group (CM group); astaxanthin pretreatment group (AST+CM group); N-acetylcysteine pretreatment group (NAC+CM group). After the cells received different intervention for the indicated time, DAPI DNA fluorescence staining detected cells apoptosis; Annexin-V-FITC / PI dual-labeled flow cytometry was used to detect the apoptosis rate; the levels of Reactive oxygen species (ROS) was detected by Flow cytometry; the protein levels of NLRP3 and CASPASE1 were detected by Western Blotting; the levels of IL-1 β and IL-18 was detected by ELISA.**RESULTS:** The measurement results show a significant increase ($P < 0.05$) in the levels of ROS, NLRP3, CASPASE1, IL-1 β , IL-18 and apoptosis rate in the CM group compared with the CON group. Compared with the CM group, a significant improvement in these unfavorable parameters was observed in AST+CM group and NAC+CM group. There was no significant difference in the above parameters between AST+CM group and NAC+CM group ($P > 0.05$).**CONCLUSIONS:** Astaxanthin can attenuate iohexol-induced human proximal renal tubular epithelial cells injury, and its possible mechanism is related to the inhibition of ROS production and down-regulation of NLRP3 inflammasome and its downstream apoptosis and inflammatory response.**Key words:** Astaxanthin, CI-AKI, ROS, NLRP3 inflammasome, apoptosis, inflammatory

INTRODUCTION

With the wide clinical application of coronary interventional techniques, especially in elderly patients with severe comorbidities, contrast-induced acute kidney injury (CI-AKI) is the third leading cause of hospital-acquired renal failure[1]. CI-AKI not only prolongs hospital stays, increases the financial burden on patients, but also increases long-term complications and mortality[2, 3]. However, there are currently no effective prevention and treatment measures in clinical practice. Therefore, early diagnosis, early prevention, and finding new drugs to treat CI-AKI are crucial. The occurrence of CI-AKI is the result of multiple factors. Studies had shown that the incidence of CI-AKI is mainly related to contrast media(CM) leading to renal microvascular hemodynamic changes, direct toxicity of CM to renal tubular cells, oxidative stress, apoptosis, inflammatory injury and tubular obstruction[4-6].

Astaxanthin (AST) is a xanthophyll carotenoid of predominantly marine origin, with potent antioxidant, anti-apoptosis and anti-inflammatory effects demonstrated in both experimental and human studies. Many studies have proven that astaxanthin has a preventive effect on various kidney diseases[7-10]. Oxidative stress, apoptosis and inflammatory injury are common pathophysiological features of CI-AKI, hence AST may have a potential therapeutic role in this condition. In the current study, we proposed to investigate the effect of Reactive oxygen species (ROS) inhibition on iohexol-induced renal tubular epithelial cell injury and the underlying mechanism through evaluating the NLRP-3 inflammasome-associated protein and the downstream inflammatory factors in HK-2. And further verify that the mechanism of astaxanthin ameliorate iohexol-induced renal tubular epithelial cell injury is through the ROS/NLRP3 pathway.

MATERIALS AND METHODS

Materials and reagents

Astaxanthin (sigma, USA); Iohexol(Yangzijiang , China); N-Acetyl-L-cysteine(Aladdin, China); Fetal bovine serum(Sijiqing, China); DMEM/F12 me-

dium(Hyclone, USA); penicillin/streptomycin (Beyotime, Shanghai, China); ROS Assay Kit (Beyotime, China); BCA Protein Kit, AnnexinV-FITC/PI Apoptosis Kit (Kaiji Biotechnology, China); DAPI (Invitrogen Biotechnology, USA); enhanced chemiluminescent detection kit (Shanghai Tianneng Technology Co., Ltd.); Radio Immunoprecipitation Assay (RIPA) Lysis Buffer (Beyotime, China); anti-NLRP3(CST, USA); anti-CASPASE1 (Proteintech, USA); anti- β -actin(Proteintech, USA); IL-1 β enzyme-linked immunosorbent assay (ELISA) kit and IL-18 enzyme-linked immunosorbent assay (ELISA) kit (ABclonal, China).

Cell culture and grouping

Human proximal renal tubular epithelial cells (HK-2) were purchased from the Chinese Type Culture Collection (CTCC, Shanghai, China), and were maintained in DMEM/F12 medium supplemented with 0.1% of a penicillin/streptomycin mixture and 10% fetal bovine serum. The cells were routinely grown in 10cm cell culture plates at 37°C in a humidified atmosphere with 5% carbon dioxide. When the cells grow to 70%~80%, continue to culture the cells for 24 h in serum-free medium, which will synchronize the cells to the stationary growth phase (G0/G1 phase). Then randomly divided the cells into 6 groups: blank control group (Control group); Dimethyl sulfoxide solvent control group (DMSO group): Add dimethyl sulfoxide to the medium to a concentration of 0.1%; astaxanthin control group (AST group): add AST to the medium to a concentration of 20 μ mol /L; Contrast media group (CM group): HK-2 cells were cultured in medium containing 100g/L of iohexol for 24 h; astaxanthin pretreatment group (AST+CM group): HK-2 cells were pretreated with medium containing 5 mM AST for 18 h, and treated with medium containing 20 μ M AST and 100g/L iohexol for 24h; N-acetylcysteine pretreatment group (NAC+CM group): HK-2 cells were pretreated with medium containing 5 mM N-acetylcysteine for 1 h, and treated with medium containing 5 mM N-acetylcysteine and 100g/L iohexol for 24 h.

DAPI DNA fluorescent staining

Lightly wash HK-2 cells grown in 6-well culture plates with PBS solution 3 times, fixed cells in 4% paraformaldehyde for 15 min at room temperature, and then gently washed 3 times with PBS solution for 5 min. DAPI (4,6-diamidino-2-phenylindole, fluorescent DNA binding dye) was added to the cell culture plate for 5-10 min in the dark, and then washed 3 times with PBS solution for 5 min. The cells were observed on an Olympus fluorescence microscope with excitation light at 400 nm and emission at 455 nm.

Annexin V-Fluorescein Isothiocyanate Conjugated Propidium Iodide Staining

Gently rinsed the cells in the plates with 2 ml of PBS solution, and then removed 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 μ l of cell suspension (5×10^5 cells) into a clean centrifuge tube, centrifuge at $1000g \times 5$ min, and then remove the supernatant. The cells were washed once with 500 μ l of PBS, and the supernatant was removed after centrifugation. Then, 500 μ l of 1×Binding Buffer was added to wash the cells again, and the supernatant was removed after centrifugation. After resuspending the cells with 100 μ l of 1×Binding Buffer, 5 μ l of Annexin V-APC was added to react at room temperature for 15 min in the dark. The cells were washed by adding 500 μ l of 1× Binding Buffer, and the supernatant was removed after centrifugation. After resuspending the cells by adding 200 μ l of 1× Binding Buffer, 5 μ l of PI was added. The samples were analyzed by flow cytometry within 1 hour.

Western blotting

The cells were harvested using Radio Immunoprecipitation Assay (RIPA) Lysis Buffer. Cell lysates (10 μ g) were electrophoresed on polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with TBS containing 3% bovine serum albumin and 0.1% Tween-20, and afterward incubated with the primary antibody and secondary antibodies and then analyzed using an enhanced chemiluminescent detection kit. Semi-quantitative Western-blot

analysis was conducted through measuring the Optical Densitometry in three independent experiments using ImageJ software.

ELISA

The cells were digested with trypsin and collected in a sterile tube, then centrifuged for about 20 minutes (2000-3000 rpm). Collect the supernatant carefully. The supernatant was carefully collected and diluted with PBS to a cell concentration of about 10^6 cells/ml. The cells are destroyed by repeated freeze-thaw cycles and the intracellular components are released. Centrifuge for about 20 minutes (2000-3000 rpm). Collect the supernatant carefully. The levels of IL-1 β and IL-18 were detected with ELISA kits according to the manufacturer's instructions. The absorbance was measured by a microplate reader.

Flow cytometry for detection of intracellular ROS levels

The cells in the logarithmic growth phase were digested, centrifuged, counted on a cell counting plate, and the cell seeding density was 4×10^5 cells/ml. The cells were seeded in a 6-well plate and cultured for 24 hours until the cells were attached. The cells were treated with the corresponding drugs according to the group: the negative control group had no special treatment; the positive control group was added with Rosoup 1 μ l +1 ml DCFH-DA; after the treatment time of each group, except for the negative control group, each experimental group was added 1ml fluorescent probe DCFH-DA(10 μ mol/ L). After culturing the cells for 30 min at 37 ° C in a 5% CO₂ incubator, the medium was discarded, and washed with serum-free medium for 3 times \times 5 min to completely remove DCFH-DA which did not enter the cells. The cells were digested with trypsin and collected, and the cells were placed in a flow cytometer to measure ROS levels. The excitation light of the flow cytometer was set to 488 nm, and the emitted light was set to 525 nm.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 software. All data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). The rate was compared

using the χ^2 test, and the t-test was used for comparison between the two groups. The comparison between the data of multiple groups was analyzed by one-way ANOVA. $P < 0.05$ indicates that the difference was statistically significant.

RESULTS

Detection of Proliferation Activity by CCK8

The results of CCK-8 showed (Figure 1): Compared with the Control group, there was no significant difference in the DMSO group and the AST group; the proliferation activity of the CM group was significantly decreased ($P < 0.05$). The proliferation activity in the AST+CM group and the NAC+CM group were significantly higher ($P < 0.05$) than that in CM group. There was no significant difference in cell proliferation activity between the AST+CM group and the NAC+CM group.

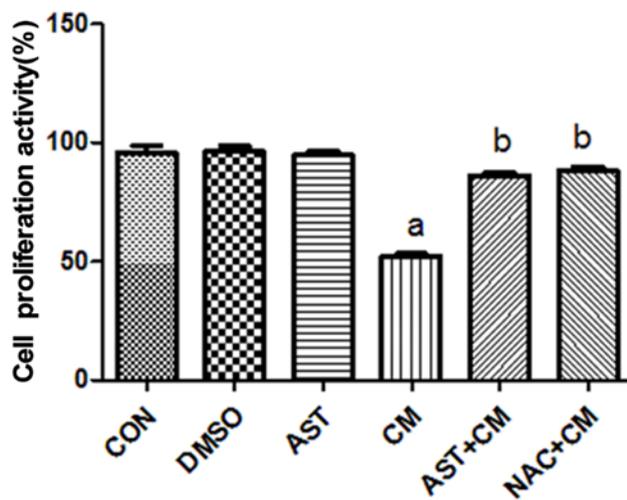


Figure 1. Comparison of proliferation activity of HK-2 cells in each group (n=5)

^a $P < 0.05$ versus the Control group; ^b $P < 0.05$ versus the CM group

Detection of Expression Levels of NLRP3 and CASPASE1 by Western Blotting

Western blotting showed that there was no significant difference between DMSO, and AST group compared with Control group; NLRP3 and CASPASE1 protein expression significant increase in CM group ($P < 0.05$). The expression of NLRP3 and CASPASE1 protein was significantly decreased after AST pretreatment ($P < 0.05$). After administration of ROS in-

hibitor NAC, the content of NLRP3 and CASPASE1 protein was also significantly decreased ($P < 0.05$). (Figure 2).

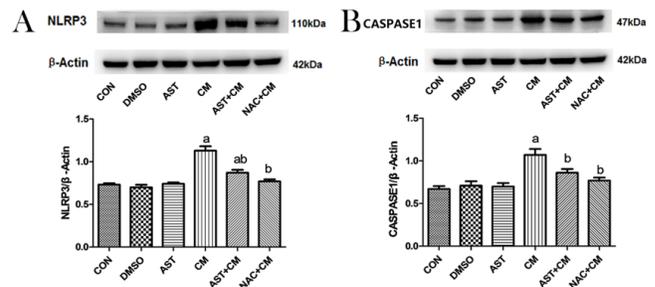


Figure 2. Protein expression of NLRP3 and CASPASE1 in HK-2 cells of each group (n=3)

(A) Representative western blot analysis of the protein expression of NLRP3. (B) Representative western blot analysis of the protein expression of CASPASE1. β -Actin was used as the loading control. Data were normalized to the expression level of β -Actin. ^a $P < 0.05$ versus the Control group; ^b $P < 0.05$ versus the CM group

DAPI fluorescent staining

In this experiment, the morphology of the nucleus was observed by DAPI DNA fluorescent staining. DAPI can be combined with the A-T base of double-stranded DNA. After DAPI staining, the nucleus will emit pale blue-white fluorescence. The nucleus of normal cells is approximately circular, with clear edges, uniform staining and light staining. Early apoptotic cells showed nuclear pyknosis and nuclear staining deepened; late apoptotic cells showed a nuclear fragmentation into circular bodies of different sizes, which were surrounded by cell membranes, i.e. apoptotic bodies, and the DAPI staining fluorescence of the nucleus was stronger. The experimental results are shown in Figure 3A: Control group, DMSO group and AST group, the nuclear staining was uniform, no apoptotic cells were observed. The cells in the CM group were inferior to the control group. The nucleus was pyknotic and the nucleus was deeply stained. The condensed nucleus was highlighted, and some cells showed nuclear lysis, which was apoptotic cells. Compared with the CM group, the nucleus pyknosis, nuclear deep staining, and apoptotic cells were reduced after pretreatment with ROS inhibitor NAC. Similar to the results of the NAC+CM group, apop-

otic cells were significantly reduced in the AST-treated group.

Detection of Apoptosis rate by Annexin V /PI

Compared with the Control group, there was no significant difference in the DMSO group and the AST group; while, the apoptosis rate of the CM group was significantly increased ($P<0.05$). The apoptosis rates of the AST+CM group and NAC+CM group were significantly lower than that of CM group ($P<0.05$). There was no significant difference between AST group and NAC group ($P>0.05$). (Figure 3B)

3A

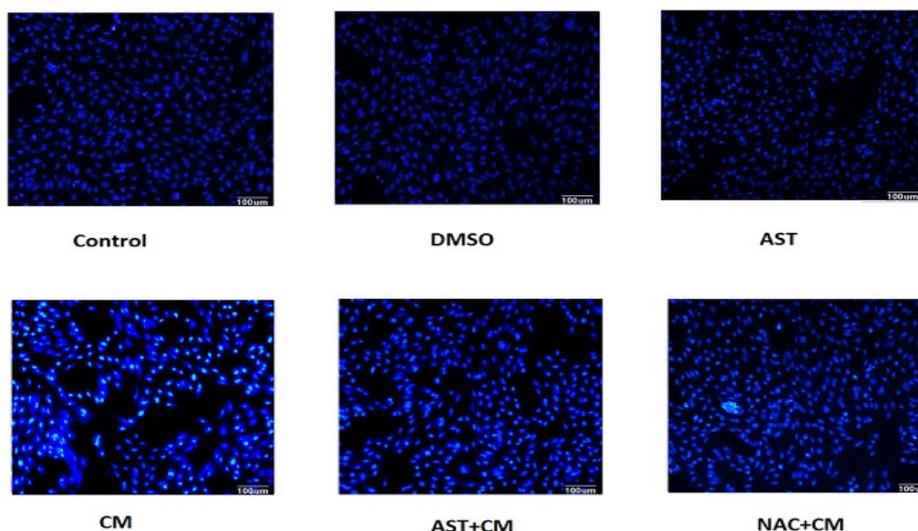


Figure 3A. DAPI fluorescence staining of HK-2 cells in each group ($\times 200$)

Control group, DMSO group and AST group, the nuclear staining was uniform, no apoptotic cells were observed. The cells in the CM group were inferior to the control group. The nucleus was pyknotic and the nucleus was deeply stained. The condensed nucleus was highlighted, and some cells showed nuclear lysis, which was apoptotic cells. Compared with the CM group, the nucleus pyknosis, nuclear deep staining, and apoptotic cells were reduced after pretreatment with ROS inhibitor NAC. Similar to the results of the NAC+CM group, apoptotic cells were significantly reduced in the AST-treated group.

3B

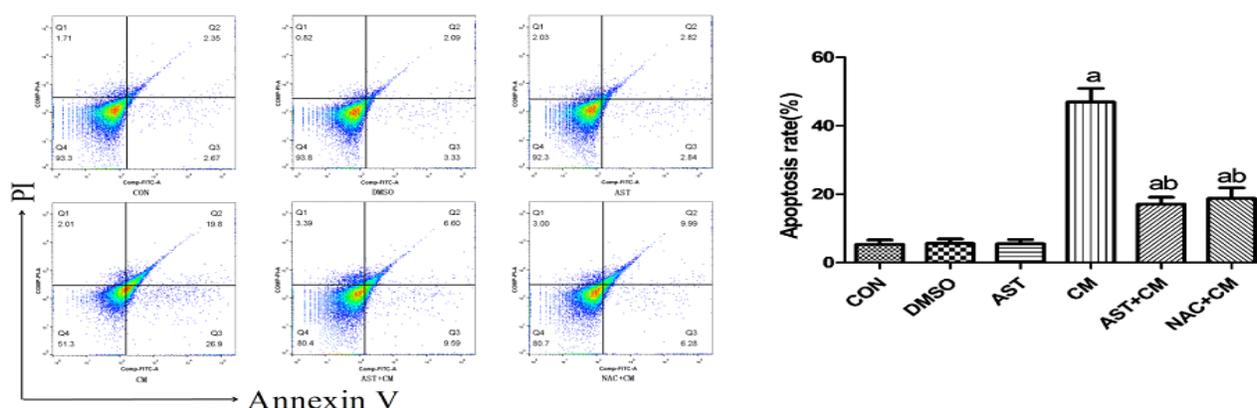


Figure 3B. Apoptosis rate detected by flow cytometry after Annexin V /PI staining ($n=3$)

The apoptosis rate is the sum of Q2 and Q3. Q2, Annexin V+/PI+ represents the rate of late apoptotic cells; Q3, Annexin V+/PI- represents the rate of early apoptotic cells; Q4, Annexin V-/PI- represents the rate of living cells. FITC, fluorescein isothiocyanate; PI, propidium iodide. ^a $P<0.05$ versus the Control group; ^b $P<0.05$ versus the CM group.

Detection of Expression Levels of IL-1 β and IL-18 by ELISA

Compared with the Control group, there was no significant difference in the DMSO and AST groups; while, the expression levels of IL-1 β and IL-18 in the CM group were significantly increased ($P<0.05$). The expression levels of IL-1 β and IL-18 in the AST+CM group and the NAC+CM group was significantly decreased ($P<0.05$). However, there was no significant difference between the AST+CM group and the NAC+CM group. (Figure 4).

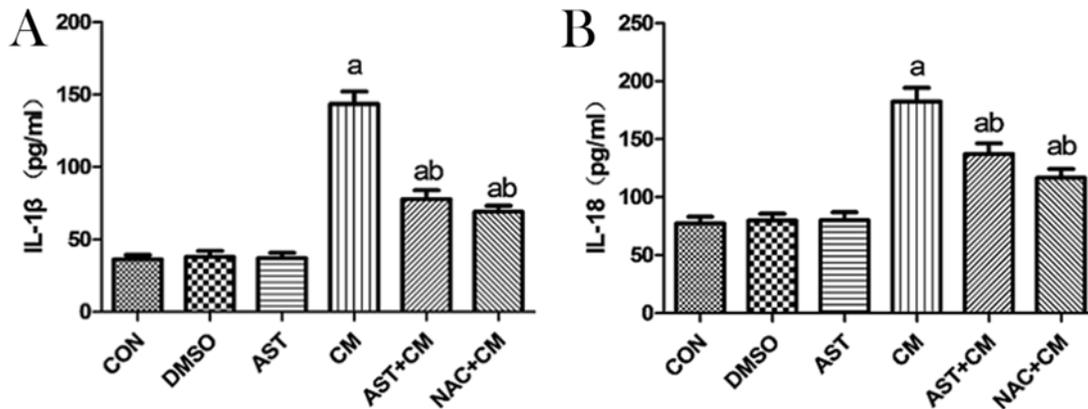


Figure 4. Expression levels of IL-1 β and IL-18 in HK-2 cells of each group(n=3)

The expression levels of IL-1 β and IL-18 was detected by ELLISA Kits. Results are presented relative to the value of the CON group and the CM group samples. ^a $P<0.05$ versus the Control group; ^b $P<0.05$ versus the CM group.

Detection of Expression Levels of ROS by Flow cytometry (FCM)

ROS, including all unstable products produced by O₂ metabolism, such as super active superoxide anion $\cdot\text{O}_2^-$, HO \cdot and non-free radical molecules H₂O₂. They are produced by aerobic metabolism, and the production of ROS increases when oxidative stress occurs in the body. Compared with the Control group, there was no significant difference in the DMSO group and the AST group; while, the ROS content in the CM group was significantly increased ($P<0.05$). The ROS level of AST+CM group and NAC+CM group was significantly lower than that of CM group ($P<0.05$). The results show that both AST pretreatment and NAC pretreatment can reduce the production level of ROS in HK-2 cells. (Figure 5)

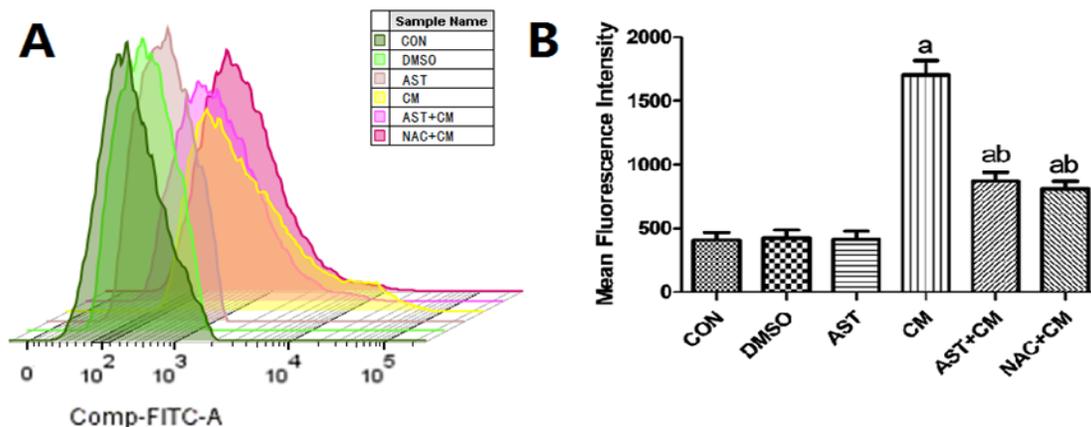


Figure 5. Reactive oxygen species levels in each group of HK-2 cells(n=3)

(A) The flow histogram of ROS was detected by Flow cytometry; (B) Reactive oxygen species levels in each group of HK-2 cells. Results are presented relative to the value of the CON group and the CM group samples. ^a $P<0.05$ versus the Control group; ^b $P<0.05$ versus the CM group. FITC, fluorescein isothiocyanate.

DISCUSSION

Contrast media, might giving rise to renal hypoxic-ischemic, oxidative stress or direct cytotoxicity of itself and so on, has been supposed to be a causative and aggravating factor of progressive AKI[11-13]. The occurrence of CI-AKI increases the medical burden of patients. Some serious patients will progress to chronic renal failure, and some even need renal replacement therapy, which seriously threatens the prognosis of patients. Hydration therapy is currently recognized as an effective measure to prevent CI-AKI, and is a simple, effective and economical method for preventing CI-AKI[14]. However, for patients with advanced age, hypertension, and heart and renal dysfunction, the use of hydration therapy 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 [15]. Therefore, the research of drugs has gradually become a research hotspot of CI-AKI prevention. The results of this study showed that AST exerts a renal protective effect on CI-AKI by increasing cell proliferation activity, alleviating apoptosis, and releasing inflammatory factors such as IL-1 β and IL-18.

Recent studies have shown that NLRP3 inflammasome are closely related to the innate immune response of the body. Multiple pathogen-associated molecular patterns and risk-related molecular patterns can activate inflammasome, cause the production of pro-inflammatory cytokines such as IL-1 β and provoke apoptosis [16, 17]. Emerging studies have shown that NLRP3 inflammasome plays an important role in the development of CI-AKI in vivo and in vitro studies, and mediates downstream apoptosis and inflammatory responses[18, 19]. It is well known that the mechanism of CI-AKI is closely related to oxidative stress, apoptosis and inflammatory response[20, 21]. It has been reported that oxidative stress plays an important role in CI-AKI[22, 23]. Increased production of ROS can induce inflammatory responses by increasing the direct or indirect expression of pro-inflammatory cytokines and chemokines. Reactive oxygen species (ROS) is currently recognized as one of the key molecules that activate the NLRP3 inflam-

matory complex[24-26]. However, whether NLRP3 inflammatory body-associated protein and its downstream inflammatory factors are regulated by ROS in CI-AKI or not is unclear yet. Our in vitro cells-based study showed that ROS expression is up-regulated and NLRP3 inflammasome was associated with cell apoptosis and inflammatory response in CI-AKI. Combined with the results after inhibiting the expression of ROS by NAC, the inhibitor of ROS, the study confirmed that ROS can activate NLRP3 inflammasome and its downstream apoptosis and inflammatory response.

Astaxanthin (AST) is a naturally occurring red carotenoid derivative widely found in many animals and plants, especially marine organisms such as microalgae, salmon, shrimp, squid, crab, etc. The special structure of AST imparts superior antioxidant activity to it, which is hundreds of times more reductive than vitamin E. AST is the strongest antioxidant found in nature to date. Recent researches demonstrated that the antioxidant activity of AST plays a protective role in acute kidney injury induced by various nephrotoxic drugs, such as colistin, cisplatin and ferric nitrilotriacetate[27-29]. Studies have confirmed that astaxanthin can alleviate acute kidney injury caused by iohexol, and in the process, the decline of oxidative stress index and the regulation of apoptosis and inflammation-related proteins are observed[30, 31]. However, the connection between AST and the NLRP3 inflammasome in CI-AKI hasn't been investigated yet. Consistent with these studies about inhibition of NLRP3 inflammasome, our results showed that CM induced excess ROS generation, activated NLRP3 inflammasome in HK2 cells. AST suppressed excess ROS generation induced by CM, and significantly decreased the elevated activation of NLRP3 inflammasome as well as the cleaved CASPASE1 and IL-1 β expressions induced by CM in HK2 cells. The experimental results confirmed that pretreatment with AST can inhibit the overexpression of ROS in HK2 cells, thereby inhibiting the NLRP3 inflammasome and its downstream apoptosis and inflammatory response. The results were not statistically different from those obtained after NAC pretreatment. Therefore, we hypothesized that the pro-

protective effect of AST on iohexol-induced human proximal renal tubular epithelial cells injury might be through inhibition of ROS/NLRP3 inflammatory signaling pathway.

CONCLUSION

In conclusion, our study demonstrated that AST could attenuate the acute kidney injury induced by CM. Furthermore, the underlying mechanisms of the renal protective effects of AST were preliminarily verified through suppressing the ROS generation, alleviating the activation of NLRP3 inflammasome and its downstream apoptosis and inflammatory response. Taken together, these results suggested that AST attenuate iohexol-induced human proximal renal tubular epithelial cells injury via the ROS/NLRP3 inflammasome signal pathway.

COMPETING INTERESTS

The author(s) declare that they have no competing interests

AUTHOR'S CONTRIBUTIONS

Dongmei Gao, Shun Yao and Yanhu Sun involved in conception and design, or acquisition of data, or analysis and interpretation of data, the contributions of them are equal. Di Zheng and Quan Zhang involved in acquisition of data, or analysis and interpretation. Dongmei Gao involved in drafting the manuscript. Wenhua Li revised and approved the manuscript.

FUNDING

This study was supported by a grant from the Six talent peaks project in Jiangsu Province (2014-YY-007)

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