NEPHROLOGY - ORIGINAL PAPER

Protective effect of astaxanthin against contrast-induced acute kidney injury via SIRT1-p53 pathway in rats

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Abstract

Purpose The present study was designed to further investigate the protective effect of astaxanthin (AST) on contrast-induced acute kidney injury (CI-AKI) in rats and the relationship between SIRT1-p53 pathway and astaxanthin.

Methods 40 adult male Sprague Dawley (SD) rats were randomly divided into five groups ($n = 8$/group): control (CON), normal rats treated with AST (AST), CM-treated (CM), CM rats treated with isoform of nitric oxide synthase (iNOS) inhibitor (iNOS + CM), and CM rats treated with AST (AST + CM). Serum creatinine (Scr) and blood urea nitrogen (BUN) values were measured at 72 h following the procedure. Hematoxylin and eosin (H–E) staining was used to observe the pathologic changes of kidney. Tunel staining was used to test apoptosis of kidney tubules. Oxidative stress, SIRT1 activity, nitric oxide (NO), and 3-nitrotyrosine (3-NT) content were individually measured with the commercial available kits.

Results Compared with the CON group, Scr and BUN levels significantly increased in the CM group ($P < 0.05$), and the values in two pre-treatment groups (iNOS + CM and AST + CM) had significantly decreased ($P < 0.05$). H–E and Tunel staining had shown that renal tubular injury was severe in CM group. The renal injury score and apoptosis index in the two pre-treatment groups also decreased ($P < 0.05$). The present study showed that in CM group the levels of oxidative stress indicators significantly increased, and the activities of antioxidant stress indicators significantly decreased. These indicators in two pre-treatment groups significantly improved ($P < 0.05$). In the CM group the expression levels of SITR1 significantly increased, and the ac-p53/p53 significantly increased ($P < 0.05$). Compared with the CM group, in AST + CM group the expression levels of SIRT1 increased, the expression levels of p53 and ac-p53/p53 decreased ($P < 0.05$).The levels of NO and 3-NT in CM group significantly increased ($P < 0.05$). Compared the CM group, the levels in the two pre-treatment groups significantly decreased ($P < 0.05$).

Conclusions Astaxanthin has a protective effect on CI-AKI, the mechanism may be related to the SIRT1-p53 pathway. Astaxanthin can reduce the content of NO and 3-NT in renal tissue of CI-AKI, and alleviate the renal injury induced by contrast agents.

Keywords Acute kidney injury · Astaxanthin · Oxidative stress · Apoptosis · SIRT1-p53 pathway · 3-NT

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Introduction

With the development of invasive cardiology, especially for older patients and those with severe complications, the contrast-induced acute kidney injury (CI-AKI) has become the third leading causes of hospital-acquired renal failure [1, 2], the incidence accounting for 11%. Although the pathogenic mechanism of CI-AKI is incompletely understood, previous studies have shown that it is mainly associated with oxidative stress and the direct toxicity of contrast medium [3, 4]. AST, a novel natural and powerful antioxidant, has been proved to be efficacious in reducing the formation of reactive oxygen species in kidney, attenuating oxidative stress

and preventing renal cell injury [5, 6]. Other literatures have reported that the anti-apoptotic mechanism of SIRT1 is related to the acetylation of pro-apoptotic protein p53 [7, 8]. However whether the protective effect of AST against CI-AKI is through the SIRT1-p53 pathway still remains unknown. The present study, using rats model of CI-AKI, aim to study the protective effects of AST against CI-AKI, the relationship with SIRT1-p53 pathway and the influence of the amount of NO, 3-NT in renal tissue.

Materials and methods

Animals

Adult male S–D rats ($n = 40$), weighing 160–200 g, were obtained from Xuzhou Medical University Laboratory Animal Center (Xuzhou, China). Rats were housed in standard stainless steel hanging cages with controlled temperature (22–25 °C), humidity (35–50%), and photocycle (12 h light/dark) conditions. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published in P.R. China and with the approval of Xuzhou Medical University Ethics Committee.

Experimental design and drugs

Rats were randomly divided into five equal groups: Control (CON; $n = 8$), normal rats treated with AST (AST; $n = 8$), CM-treated (CM; $n = 8$), CM rats treated with iNOS inhibitor (iNOS + CM, $n = 8$), and CM rats treated with AST (AST + CM; $n = 8$). The AST and AST + CM groups received AST (Shanghai Yuanye Biotechnology, Shanghai, China) dissolved in olive oil via oral gavage for 10 consecutive days before the sacrifice, the other groups received the same amount of olive oil. In the seventh day, all groups were fasted for solids and water prohibition. In the eighth day, the CM, iNOS + CM and AST + CM groups, administered CI-AKI model as described by Yokomaku et al. [9], and the iNOS + CM group received iNOS inhibitor (1400 W) intraperitoneal injection 3 days continuously.

Specimen collection

Blood simples (3 ml) were harvested from the inferior vena cava of all rats at 72 h post CI-AKI. Centrifuged at $1077 \times g$ for 30 min at 4 °C and retained the supernatant. BUN and Scr values were measured by an automatic biochemical analyzer. Kidneys were harvested and separated into two pieces, one of which was reserved for pathological examination, and the other was stored at −80 °C until analysis of oxidative stress, SIRT1 activity, Western blotting, and the contents of NO and 3-NT.

Renal pathological examination

Kidneys was fixed in 10% formalin for 24 h, the fixed pieces of kidney was processed and embedded in paraffin, cut into 5 μm-thick section, and stained with H–E and Tunel. Biopsies were examined by light microscopy in a blinded manner, and scored with a semi-quantitative scale. H–E staining was designed to evaluate the degree of tubular injury, according to the tubular necrosis, medullary congestion and protein cast deposition. Tubule injury was graded on a scale: 0, normal kidney; 1, minimal damage (unicellular, patchy isolated damage); 2, mild damage (< 25%); 3, moderate damage (25–50%); and 4, severe damage (> 50%). Medullary congestion was graded on a scale: 0, no hyperemia; 1, minimal hyperemia (the congested red blood cells can be distinguished under $400 \times$ light microscope); 2, mild hyperemia (the congested red blood cells can be distinguished under $200 \times$ light microscope); 3, moderate hyperemia (the congested red blood cells can be distinguished under $100 \times$ light microscope); and 4, severe hyperemia (the congested red blood cells can be distinguished under $40 \times$ light microscope). A minimum of 10 fields at $\times 400$ magnification were assessed and graded in each biopsy. The mean of 10 fields was calculated as the injury score. Renal tubular apoptosis was evaluated by the Tunel Kit (Boster Biotech, Wuhan, China), procedure according to the instruction. Under the microscope, normal cells nuclei were observed with blue, apoptotic cells were observed with brown particles in the nucleus. Randomly selected five non-overlapping fields from each section and observed, calculated the ratio of positive cell nuclei and total cell nuclei in each field, and adopted the average as the apoptosis index (AI).

Oxidative stress

Renal tissues were homogenized and centrifuged; the supernatant was retained for determining. Renal malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-Px) levels, indicators of oxidative stress, were respectively determined under the guidance of the kits instructions (Jiangcheng Biongineering Institute, Nanjing, China).

SIRT1 acetyltransferase activity

The supernatant of renal tissues was taken to measure the protein concentration by the bicinchoninic acid assay (BCA) kit (Biyuntian Biotechnological Co., Shanghai, China). SIRT1 acetyltransferase activity was detected via the commercial available SIRT1 colorimetric assay kit (Cycle). The relative

activity of enzyme was detected by multifunctional microplate reader, contrasted with blank comparison.

Western blotting

Protein concentration was determined with BCA kit. Protein samples were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto membranes. Specific antibody SIRT1 (1:500), p53 (1:2000) and ac-p53 (1:300) (Bioss, Inc., USA) were respectively added, and incubated at 4 °C overnight. The membranes were incubated with double antibody (Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h in the dark. Protein bands were analyzed via Laser Imaging system (LI-COR Biosciences, Inc.).

Measurement of NO and 3-NT

Supernatant from kidney homogenate was prepared for detecting the levels of NO and 3-NT with commercial available NO kit (Jing Corporation) and 3-NT kit (RNA Corporation, USA). The specific procedures followed the instructions of the manufacture.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean $\pm$ standard deviation. Statistical comparisons were executed by one-way analysis of variance followed by Student–Newman–Keuls analysis. Comparison among groups were using q test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Serum Scr and BUN values

At 72 h following administration, Scr and BUN levels significantly increased in the CM group ($P < 0.05$). Compared with the CM group, the values of Scr and BUN in the iNOS + CM and AST + CM groups had significantly decreased ($P < 0.05$). As compared with the CON group, Scr and BUN values in the AST + CM had no markedly changed, there was no significant differences ($P > 0.05$) (Table 1).

Pathological changes of renal tissue

HE staining and renal injury score

In contrast-injected rats, fuzzy cortex and medullary structure, partial glomerular atrophy, vacuolar degeneration of tubular epithelial cells, tubular dilation, protein cast (protein deposition in the tubular lumen), increased epithelial cell shedding, and necrosis of partial tubular epithelial cells were observed (Fig. 1). Semi-quantitative analysis demonstrated no difference between the renal injury scores of the AST and CON groups. Renal injury scores in the iNOS + CM and AST + CM groups were higher than that of the CON group, but significantly lower than that of the CM group ($P < 0.05$, Table 2).

Tunel staining

Compared with the CON group, in the CM group Tunel positive cells significantly increased. While after the intervention of iNOS inhibitors and astaxanthin, Tunel positive cells in renal tissue obviously decreased, there were significant differences in the apoptosis index (AI) ($P < 0.05$) (Fig. 2).

Oxidative stress indicators

As shown in Table 3, a significant increase of kidney MDA was observed in CM rats compared with the CON group, and this was accompanied by an decline of T-SOD, GSH, and GSH-Px, there were significant differences ($P < 0.05$). These indicators in iNOS + CM and AST + CM groups obviously improved ($P < 0.05$). Compared with iNOS + CM group, the levels of MDA and T-SOD were lower in the AST + CM group, there were significant differences ($P < 0.05$), while there were no significant differences in the levels of GSH-Px and GSH ($P > 0.05$).

SIRT1 acetyltransferase activity

Compared with the CON group, SIRT1 activity in AST group increased, in CM group significantly decreased ($P < 0.01$). Compared with CM group, the activity in iNOS + CM and AST + CM groups obviously improved ($P < 0.05$). No significant differences were detected between the iNOS + CM and AST + CM groups ($P > 0.05$) (Fig. 3).
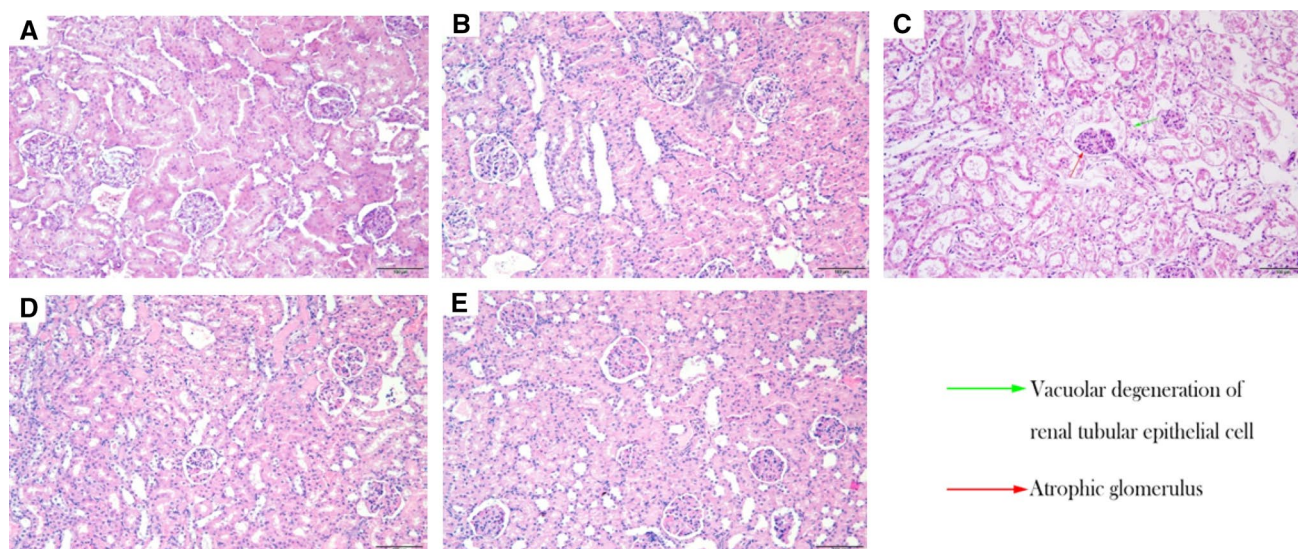
Table 1 The values of serum Scr and BUN in each group ($\bar{X} \pm s$, $n = 8$)

Groups	CON	AST	CM	iNOS + CM	AST + CM
Scr (µmol/L)	32.53 ± 1.60	30.67 ± 1.45	104.65 ± 6.82*	69.83 ± 2.21*#	40.67 ± 1.98#$
BUN (mmol/L)	5.29 ± 0.34	5.34 ± 0.37	36.80 ± 2.50*	13.04 ± 0.64*#	8.26 ± 0.40#

*$P < 0.05$ vs. the CON group; #$P < 0.05$ vs. the CM group; $$P < 0.05$ vs. iNOS inhibitor group

Fig. 1 Hemotoxylin and eosin staining of each group (magnification, ×200). **a** Control group, **b** AST group, **c** CM group, **d** iNOS + CM group, and **e** AST + CM group. As the CON and AST groups demonstrated no significant differences in staining

Table 2 The renal injury score of each group ($\bar{X} \pm s$, $n = 8$)

Groups	Epithelium necrosis	Medullary congestion	Protein cast
CON	0.05 ± 0.02	0.22 ± 0.10	0.00
AST	0.16 ± 0.12	0.20 ± 0.09	0.00
CM	$3.21 \pm 0.16^*$	$2.91 \pm 0.21^*$	$3.15 \pm 0.24^*$
iNOS + CM	$1.52 \pm 0.13^{*\#}$	$1.89 \pm 0.16^{*\#}$	$2.12 \pm 0.14^{*\#}$
AST + CM	$1.28 \pm 0.02^{\#\$}$	$1.17 \pm 0.16^{*\#\$}$	$0.92 \pm 0.20^{*\#\$}$

$^*P < 0.05$ vs. the CON group; $^{\#}P < 0.05$ vs. the CM group; $^{\$}P < 0.05$ vs. iNOS inhibitor group

Western blotting

According to the western blotting results, the expression levels of SITR1 and p53 in the kidney tissue of the CM group significantly increased, and the ratio of ac-p53 and p53 significantly increased ($P < 0.05$). Compared with the CM group, in AST + CM group the expression levels of SIRT1 increased, the expression levels of p53 and the ratio of ac-p53 and p53 decreased ($P < 0.05$). However, there were no significant differences in the p53 expression levels and ac-p53/p53 ($P > 0.05$) (Fig. 4).

NO and 3-NT levels

Compared with the CON group, the levels of NO and 3-NT in CM group significantly increased ($P < 0.05$). Compared with the CM group, the levels of NO and 3-NT in iNOS + CM and AST + CM groups significantly decreased

($P < 0.05$). No significant differences were detected between iNOS + CM and AST + CM groups ($P > 0.05$) (Table 4).

Discussion

With the development of the CI-AKI research, the pathogenesis and risk factors of CI-AKI have been recognized gradually. However, there is no an effective prevention strategy, CI-AKI is still an important issue to tackle in clinical practice. Although the mechanisms of CI-AKI are incompletely understood, the previous study indicated that renal medullary ischemic damage and the toxicity of contrast to renal tubular epithelial cells primarily contributed to the pathogenesis of CI-AKI [10]. Some studies indicated that contrast medium can enhance oxidative stress in the kidney [11–14], cause renal tubular damage and lead to apoptosis of the tubular epithelium. The present study observed that in renal tissue of CI-AKI the levels of oxidative stress markers, such as MDA significantly increased, and the activities of antioxidant stress markers, such as T-SOD and GSH-Px significantly decreased. H–E staining showed that the renal tubular lesions were serious in CM group. Therefore, CI-AKI was related to the activation of oxidative stress, and the imbalance between oxidation and antioxidant system was an important mechanism of CI-AKI.

SIRT1 is a NAD-dependent deacetylase and plays an important role in many cellular processes [15, 16]. NAD-dependent protein via deacetylate regulate transcription and participate in intercellular energy-yielding and many cellular basic functions, such as cell cycle, DNA damage, metabolism, apoptosis, and autophagy regulation. SIRT1 via
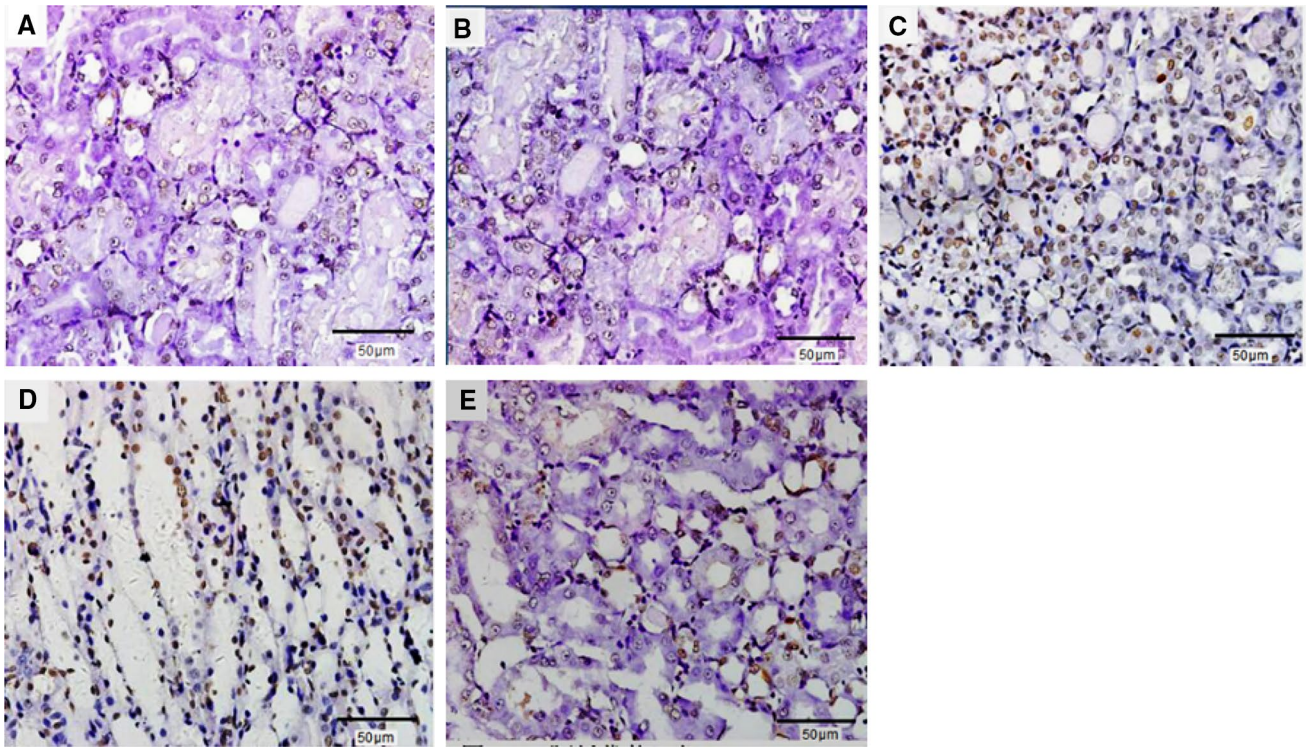
Fig. 2 Tunel staining of each group (magnification, ×400). **a** Control group, **b** AST group, **c** CM group, **d** iNOS + CM group, and **e** AST + CM group

Table 3 The indexes of oxidative stress in kidney tissue of each group ($\bar{X} \pm s$, $n = 8$)

Groups	MDA (nmol/mg protein)	T-SOD (U/mg protein)	GSH-Px (U/mg protein)	GSH (mg/g protein)
CON	1.21 ± 0.03	225.45 ± 7.10	60.46 ± 3.12	2.02 ± 0.05
AST	1.24 ± 0.02	234.14 ± 9.31	58.04 ± 1.66	2.12 ± 0.08
CM	$1.60 \pm 0.03^*$	$152.11 \pm 4.34^*$	$43.54 \pm 2.93^*$	$1.55 \pm 0.04^*$
iNOS + CM	$1.40 \pm 0.02^{*\#}$	$182.69 \pm 3.27^{*\#}$	$54.02 \pm 1.91^{\#}$	$1.83 \pm 0.04^{\#}$
AST + CM	$1.28 \pm 0.02^{\#\$}$	$207.68 \pm 4.79^{\#\$}$	$59.28 \pm 1.93^{\#}$	$1.99 \pm 0.03^{\#}$

$^*P < 0.05$ vs. the CON group; $^{\#}P < 0.05$ vs. the CM group; $^{\$}P < 0.05$ vs. iNOS inhibitor group

catalyzing histone deacetylation regulate chromatin function and promote the methylation of histone and DNA to inhibit the transcription process. Extensive deacetylation of transcription factors and co-regulatory factors can increase or decrease the expression of target genes [17]. According to the previous study, SIRTl regulate cell apoptosis via catalyzing the deacetylation of the transcription factors, such as p53, NF-K B, and FOXO. At the carboxyl end of p53, lysine residues undergo acetylation modification, then the stability, DNA binding capacity and transcription activity of P53 was enhanced. This process is regulated by SIRT1 deacetylation. SIRT1 can reduce the apoptosis of cells induced by oxidative stress by inhibiting the activity of p53 [18, 19]. SIRT1 can catalyze the deacetylation of lysine on p53 382 site, decrease p53 activity and stability, weaken the effects

of p53 on DNA damage and oxidative stress, and inhibit the transcription of p53-dependent CDKN1A and BAX. The related experiments showed that in SIRT1 transgenic mouse, the renal proximal tubular epithelium defended epithelium injury induced by cisplatin, such as apoptosis, via maintaining the number and function of peroxisomes [20]. Hao et al. [21] found that SIRT1 is highly expressed in renal medullary mesenchymal cells, if we reduced the expression of SIRT1 in cells, the cells resistance of oxidative stress decreased substantially and cell apoptosis increased. On the other hand, if we raised the expression, cell survival increased. Astaxanthin (AST) is a carotenoid pigment naturally existing in seafood, such as salmon, shells of crabs and shrimps, as well as a wide variety of plants and algae [22]. The antioxidant activity of astaxanthin is 65 times more powerful
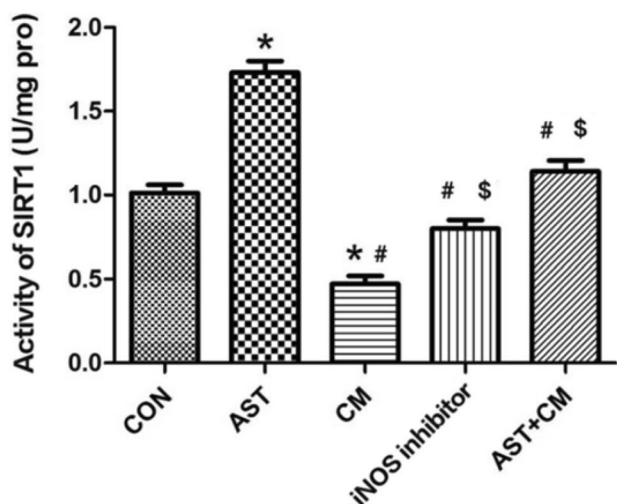
Fig. 3 SIRT1 activity in kidney tissue of each group. *$P < 0.05$ vs. the CON group; #$P < 0.05$ vs. the AST group; $$P < 0.05$ vs. CM group

than vitamin C, 54 times stronger than β-carotene, 10 times more potent than β-carotene, canthaxanthin, zeaxanthin, and lutein; and 100 times more effective than α-tocopherol [23–26]. Recent studies have shown that the astaxanthin could offer protective effects against IR-induced renal injury by reducing inflammatory, tubular apoptosis and oxygen free radical [27]. The present experiment showed that compared with the CON group, in CM group the expression levels of SITR1 and p53 significantly increased, and the ratio of ac-p53 and p53 significantly increased ($P < 0.05$). Compared with the CM group, in AST+CM group the expression levels of SIRT1 increased, the expression levels of p53 and the

ratio of ac-p53 and p53 decreased($P < 0.05$). Therefore, we can infer that AST activate SIRT1 to depress the expression and acetylation of p53, inhibit the apoptosis of renal tubular cells, and reduce renal damage.

Protein nitration belongs to post-translational modifications. The related literature confirmed that the formation of 3-NT is a special oxidation reaction, and can be used as a marker to evaluate the degree of nitrite stress. Under normal physiological conditions, nitration of tyrosine residues may occur, whereas 3-NT values are usually elevated under pathological conditions. NO was given 6 h after renal IR, it can be seen that tyrosine kinase increased, 3-NT levels elevated, and the 3-NT levels were related to the severity of the kidney, which aggravated kidney damage [28, 29]. The present study showed, in the CM group, NO and 3-NT contents increased and SIRT1 activity decreased markedly. However, compared with the CM group, NO and 3-NT contents in the AST group decreased significantly and SIRT1 activity increased. Therefore, we inferred that the protective effect of

Table 4 NO, 3-NT and apoptotic index in renal tissues of each group ($\bar{X} \pm s$, $n = 8$)

Groups	NO (μmol/L)	3-NT (nmol/L)	AI (%)
CON	1.92 ± 0.54	69.82 ± 8.56	5.25 ± 0.53
AST	1.87 ± 0.63	73.04 ± 5.30	4.71 ± 0.31
CM	$6.15 \pm 0.81^*$	$125.21 \pm 11.03^*$	$26.62 \pm 2.62^*$
iNOS+CM	$2.54 \pm 0.42^{*\#}$	$94.16 \pm 13.20^{*\#}$	$18.51 \pm 3.79^{*\#}$
AST+CM	$3.92 \pm 0.38^{*\$}$	$98.35 \pm 5.52^{*\$}$	$10.61 \pm 3.36^{*\#\$}$

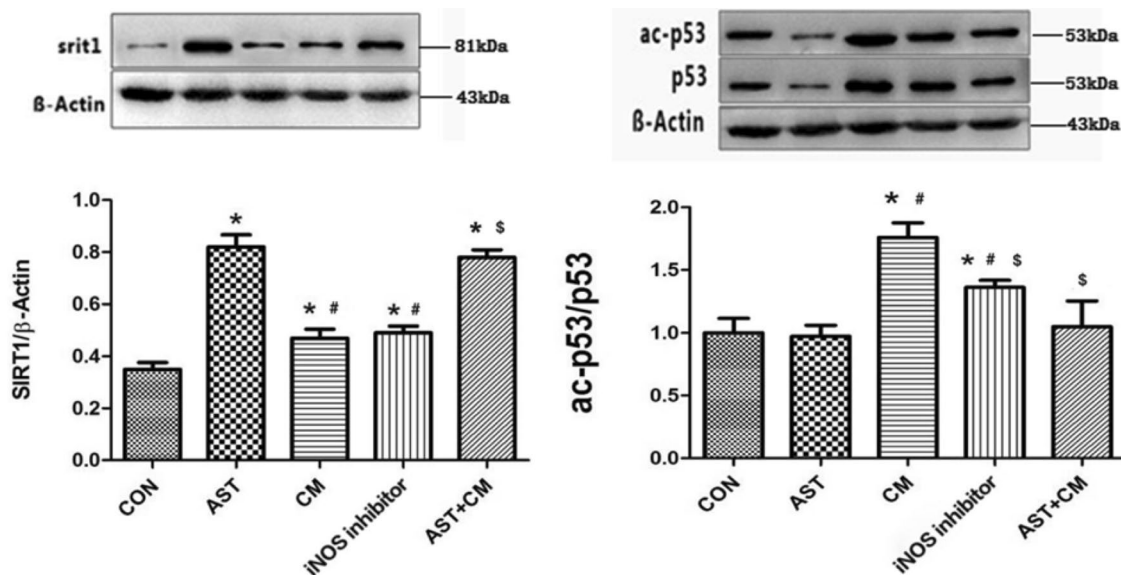*$P < 0.05$ vs. the CON group; #$P < 0.05$ vs. the CM group; $$P < 0.05$ vs. iNOS inhibitor group

Fig. 4 Expression of SIRT1, p53 and ac-p53 in renal tissue. *$P < 0.05$ vs. the CON group; #$P < 0.05$ vs. the AST group; $$P < 0.05$ vs. CM group

astaxanthin on CI-AKI, reduce oxidative damage and apoptosis of renal tubular cells, is related to inhibiting the massive production of NO and 3-NT. Meanwhile, in the iNOS group, we discovered that NO, 3-NT content decreased, at the same time there is an increase in SIRT1 activity, just like in the AST group. Therefore, we hypothesize that the SIRT1 activity, in the mechanism of the protective effect of AST on CI-AKI via SIRT1-p53 pathway reducing CI-AKI, may be related to protein nitration.

Meanwhile, many studies have shown that inflammatory factor levels are up-regulated during CI-AKI [13, 30, 31]. But is there a decreased inflammation for example of inflammatory cytokines TNF-α, IL-1β, and IL-6 after Astaxanthin treatment? This issue is still unclear so far. We assume that the mechanism of astaxanthin protection against CI-AKI might be related to down-regulation of such inflammatory factors and we will do more in-depth research to confirm this hypothesis in our subsequent experiments.

Conclusion

The present study showed that AST had protective effect on CI-AKI, the mechanism may be the up-regulation of SIRT1, which reduce the expression and acetylation of p53, inhibit the oxidative stress, and reduce cell apoptosis. In addition, SIRT1 activity is associated with protein nitration levels. While in the mechanism of AI-AKI, whether or not SIRT1 is nitrated and which sites are nitrated remain to be confirmed by further experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

Ethical approval Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published in P.R. China and with the approval of Xuzhou Medical University Ethics Committee.

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