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Original Article

Investigation of the Effect of Astaxanthin on Autophagy in Renal Ischemia-reperfusion Modeled Rats

Böbrek İskemi-reperfüzyon Modeli Oluşturulan Sıçanlarda Astaksantinin Otofaji Üzerine Etkisinin Araştırılması

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ABSTRACT

Objective: The aim of this study was to investigate the effect of various astaxanthin (ATX) doses on oxidative damage and autophagy in renal ischemia-reperfusion (I/R) injury-modeled rats.

Methods: The rats were divided into five groups: sham group (n=8), I/R (n=8), I/R + 5 mg/kg ATX (n=8), I/R + 10 mg/kg ATX (n=8), and I/R + 25 mg/kg ATX (n=8) groups. ATX was dissolved in 5 mg/kg, 10 mg/kg, and 25 mg/kg olive oil for 7 days and administered to the rats in the experimental group. Sham and I/R groups were also administered ATX solution (olive oil) via oral gavage for 7 days. Renal ischemia reperfusion was induced in all rats except the sham group after the last dose was administered on the 7th day. Reperfusion was conducted for 24 hours after 45 minutes of ischemia.

Results: Blood samples were collected, and kidney tissue were incised for biochemical and histological analyses. Superoxide dismutase (SOD) and total antioxidant status (TAS) were significantly lower in the I/R group than in the sham group ($p<0.05$), whereas malondialdehyde (MDA) and total oxidant status (TOS) values were higher ($p<0.05$). It was determined that SOD and TAS increased and MDA and TOS decreased in the ATX-administration groups compared with the I/R group, independent of the dose ($p<0.05$). In the 25 mg/kg ATX + I/R group, Beclin-1 and LC3β immunoreactivities were significantly higher than those in the other groups ($p<0.05$). The lowest p62 immunoreactivity was observed in the 25 mg/kg ATX + I/R group.

Conclusions: ATX had a protective effect on kidney function and against oxidative damage. Furthermore, high-dose ATX administration protected kidney tissue via autophagy induction in this study.

Keywords: Astaxanthin, renal, ischemia/reperfusion, oxidative stress, autophagy

ÖZ

Amaç: Bu çalışmanın amacı böbrek iskemi-reperfüzyon (İ/R) hasarı olan sıçanlarda astaksantinin (ATX) otofaji üzerine etkisinin araştırılmasıdır.

Yöntemler: Sıçanlar 5 gruba ayrıldı: Sham grubu (n=8), İ/R (n=8), ATX 5 mg/kg + İ/R (n=8), ATX 10 mg/kg + İ/R (n=8), ATX 25 mg/kg + İ/R (n=8). Deney grubundaki sıçanlara zeytinyağında çözdürülmüş ATX 5 mg/kg, 10 mg/kg ve 25 mg/kg, Sham ve İ/R grubundaki sıçanlara ise ATX çözücüsü (zeytinyağı) gavaj yoluyla 7 gün boyunca verildi. Sham grubu dışındaki tüm sıçanlara 7. gün son doz uygulandıktan sonra İ/R uygulandı. Kırk beş dakikalık böbrek iskemisinden sonra 24 saat süreyle reperfüzyon yapıldı.

Bulgular: Biyokimyasal ve histolojik analizler için kan ve doku örnekleri toplandı. Sham grubunda göre İ/R grubunda süperoksit dismutaz (SOD) ve toplam antioksidan durumu (TAS) anlamlı olarak düşükken ($p<0,05$), malondialdehid (MDA) ve toplam oksidan durumu (TOS) değerleri daha yüksekti ($p<0,05$). ATX uygulanan gruplarda doz miktarından bağımsız olarak SOD ve TAS düzeylerinin arttığı, MDA ve TOS düzeylerinin azaldığı tespit edildi ($p<0,05$). ATX 25 mg/kg + İ/R grubunda Beclin-1 ve LC3β immünreaktiviteleri diğer gruplarla karşılaştırıldığında istatistiksel olarak anlamlı derecede yüksekti ($p<0,05$). En düşük p62 immünreaktivitesi ATX 25 mg/kg + İ/R grubunda tespit edildi ($p<0,05$).

Sonuçlar: ATX'in böbrek fonksiyonlarını ve oksidatif hasara karşı koruyucu etkisi vardı. Ayrıca bu çalışmada yüksek doz ATX uygulaması böbrek dokularını otofaji indüksiyonu yoluyla korumuştur.

Anahtar kelimeler: Astaksantin, böbrek, iskemi/reperfüzyon, oksidatif stres, otofaji

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INTRODUCTION

The kidney is an organ susceptible to ischemia-reperfusion (I/R) damage. Renal ischemia is the major cause of acute kidney injury induced by various conditions such as low cardiac output, renal vascular occlusion or obstruction, sepsis, and kidney transplantation. Acute kidney injury can lead to morbidity and mortality, affecting approximately 5-30% of inpatients and intensive care patients[1].

I/R injury induces certain structural and metabolic changes in tissues[2]. Thus, tissues require a defense mechanism to maintain vitality. Antioxidant defense is one of these mechanisms. There is a balance between the oxidant and antioxidant systems. An increase in the oxidant level and inadequate antioxidant system leads to oxidative damage[3]. There are various antioxidants in nature. Astaxanthin (ATX) is a red-orange antioxidant in the ketone group of carotenoids[4]. The effects of ATX on living organisms include the promotion of the immune system, anti-inflammatory properties, and protection against neurodegenerative diseases[5]. Certain studies have emphasized the protective effect of ATX against I/R injury[6-8].

Autophagy is a catabolic event that literally means self-eating. It is observed at the basal level in most cells and promotes the transformation of perennial organelles and proteins to maintain intracellular homeostasis[9]. The induction of autophagy during cellular stress is an effective mechanism for cellular survival. Certain empirical studies have reported that the inhibition of autophagy increases kidney damage, demonstrating the protective effect of autophagy against ischemic kidney damage[5]. Various studies have demonstrated that activation of autophagy triggered necrosis pathways and increased kidney damage[10]. Induction of autophagy increases Beclin-1 and LC3β protein activities, whereas it decreases p62 protein activity. Thus, Beclin-1, LC3β and p62 proteins are among the most frequently used parameters to analyze autophagy[11-13].

ATX has a protective effect against oxidative damage due to its antioxidant properties. Furthermore, it could induce and inhibit the autophagy mechanism. The role of autophagy in I/R-induced kidney damage has not yet been clarified. Thus, this study investigated the effects of various ATX doses on oxidative damage and autophagy in renal I/R injury modeled rats.

MATERIALS and METHODS

Study Settings

This study was approved (protocol no: 2019/A-48, date: 23.10.2019) by Inonu University, Animal Experiments Local Ethics Committee. During the study, the experimental animals were cared for and all experimental procedures conducted at Inonu University Faculty of Medicine Experimental Animal Breeding and Research Center.

Experimental Animals and Groups

This study was conducted with 40 male Sprague-Dawley rats with an average weight of 250 g. During the experiments, animals were kept at 21±1 ˚C temperature and 12 hours (h) of light and 12 h of darkness and fed standard rat chow ad libitum and regular tap water. The rats were divided into five groups: sham group (n=8), I/R (n=8), I/R + 5 mg/kg ATX (n=8), I/R + 10 mg/kg ATX (n=8), and I/R + 25 mg/kg ATX (n=8) groups. ATX[14] (Galenik, Cas no: 742-61-7) was dissolved in 5 mg/kg, 10 mg/kg, and 25 mg/kg[15] olive oil for 7 days[16] and administered to the rats in the experimental group. Sham and I/R groups were also administered ATX solution (olive oil) via oral gavage for 7 days. Renal ischemia reperfusion was induced in all rats except the sham group after the last dose was administered on the 7th day. Reperfusion was conducted for 24 h after 45 minutes (min) of ischemia. After 24 h, blood samples were drawn from the animals. The rats were then decapitated. Blood samples were collected for blood urea nitrogen (BUN) and creatinine measurements, and kidney tissue were incised for biochemical and histological analyses.

Rat Kidney I/R Damage Model

All rats were anesthetized with intramuscular 70 mg/kg ketamine (Richter Pharma AG, Australia) and 8 mg/kg xylazine (Bioveta PLC, Czech Republic) administration. Both renal arteries were revealed in the abdominal wall of the rats. A clamp was attached to the renal arteries, and ischemia was induced by obstructing blood flow to the kidneys for 45 min[17]. After 45 min of ischemia, the renal artery clamps were removed and blood flow was restored to the kidneys. After 24 h of reperfusion, the rats were sacrificed under anesthesia, and blood samples and kidney tissue samples were collected.

Blood Urea Nitrogen and Creatinine Measurements

BUN and creatinine levels were measured to determine the kidney function of the experimental group. Analyses were conducted based on the protocol described by commercial ELISA kits (SunRed Biotechnology, China)

with a microplate reader and BioTek HT Synergy Gen 5 software.

Superoxide Dismutase and Malondialdehyde Measurements

Kidney tissue superoxide dismutase (SOD) enzyme activity was determined using the method described by Sun et al.[18]. Malondialdehyde (MDA) was measured using the Esterbauer and Cheeseman[19] method, the most common lipid peroxidation determination method.

Total Antioxidant Status and Total Oxidant Status

The kidney tissue total antioxidant status (TAS) and total oxidant status (TOS) were determined based on the protocol provided in commercial ELISA kits (SunRed Biotechnology, China) with a microplate reader and BioTek HT Synergy Gen 5 software in the experimental groups.

Histochemical Analysis

The kidneys were removed at the end of the experiment and fixed in 10% formaldehyde. After tissue follow-up procedures, 4-5 µm sections were incised from the paraffin blocks. Sections were stained with hematoxylin-eosin (H&E) to determine the general morphological structure. Kidney sections were evaluated for tubular degeneration (tubular necrosis and dilatation) in the cortical and medullary regions. Ten randomly selected areas were examined, and the regions were scored on the basis of the degree of histological changes as follows: 0: no change, 1: mild, 2: moderate, 3: severe change. Analyses were performed using a Leica DFC-280 research microscope and the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Immunohistochemical Analysis

The deparaffinized and rehydrated sections were placed in a pressure cooker and boiled in 0.01 M citrate (pH 6.0) for 15-20 min. To inhibit endogenous peroxidase enzyme activity, 3% hydrogen peroxide was applied to the sections for 12 min. A protein block (ultra V block) was applied to the sections that were washed with phosphate buffered saline (PBS) for 5 min. The sections were then incubated with primary antibody (GnRH and kispeptin) at 37 °C for 60 min. Biotin-based secondary antibody was applied to the tissues that were washed with PBS for 10 min at 37 °C. The sections were then incubated at 37 °C for 10 min with streptavidin peroxidase. Subsequently, chromogen-applied sections were stained with hematoxylin and covered with a water-based sealer. Brown staining was observed in tubule epithelial cells because of the immunoreactivity with Beclin-1, LC3β and p62. Staining, extent of immunoreactivity (0: 0-25%, 1: 26-50%, 2: 51-75%, 3: 76-100%), and severity (0: none, +1: mild, +2: moderate, +3: severe) were scored semi-quantitatively. The total staining score was calculated by 'prevalence x severity'[20]. The analysis was conducted using a Leica DFC-280 research microscope and a Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical Analysis

Statistical analyses were conducted using the Kruskal-Wallis test on IBM SPSS Statistics 25.0 software. The Conover test was used for Kruskal-Wallis in multiple comparisons. $p<0.05$ was accepted as statistically significant. Data are presented as medians (minimum-maximum). Statistical histological analyses were conducted using statistical software developed by Inonu University Faculty of Medicine, Department of Biostatistics and Medical Informatics. It was determined that measurable variables in all study groups did not exhibit a normal distribution in the normality tests. Thus, Kruskal-Wallis analysis of variance, a non-parametric test, was employed in the general intergroup comparisons of all variables. Paired-group comparisons were determined using the Mann-Whitney U test and Bonferroni correction. $P<0.05$ was accepted as statistically significant. Data are presented as medians (minimum-maximum) based on distribution.

RESULTS

Astaxanthin Preserves Kidney Function

BUN and creatinine levels were measured to determine rat kidney functions. BUN and creatinine levels were significantly higher in the I/R group than in the sham group ($p<0.05$). BUN and creatinine levels were found to be significantly lower in animals given ATX and performed I/R than in the I/R group ($p<0.05$) (Table 1).

Astaxanthin Protects Against Oxidative Stress

SOD, MDA, TAS, and TOS were measured to determine oxidative stress in kidney tissue. SOD and TAS were significantly lower in the I/R group than in the sham group ($p<0.05$), whereas MDA and TOS values were higher ($p<0.05$). It was determined that SOD and TAS increased and MDA and TOS decreased in the ATX-administration groups compared with the I/R group, independent of the dose ($p<0.05$) (Table 1).

Histopathological Changes in Kidney Tissues

The kidney tissues of the sham group had normal histological appearance, except for mild tubular dilatation. However, necrotic changes and dilatation were observed in both cortical and medullar tubules in the I/R group. Another prominent finding in this group was local and diffuse inflammatory cell infiltration in the interstitial tissues. The difference between the I/R and sham groups was statistically significant ($p<0.0001$). It was observed that the histopathological changes observed in the 5 mg/kg ATX + I/R group were similar to those in the I/R group. The histopathological changes observed in the I/R group were significantly lower in the 10 mg/kg ATX + I/R and 25 mg/kg ATX + I/R groups ($p<0.0001$). The most significant decrease in infiltration was observed in the 25 mg/kg ATX + I/R group (Figure 1, Table 2).

Astaxanthin Induces Autophagy

Immunoreactivity of Beclin-1, LC3β and p62 proteins was analyzed to investigate autophagy. Beclin-1 and LC3β immunoreactivities were prominent in the tubule epithelial cell cytoplasm. Statistically, Beclin-1 and LC3β immunoreactivities were similar in the sham, I/R, 5 mg/kg ATX + I/R, and 10 mg/kg ATX + I/R groups. In the 25 mg/kg ATX + I/R group, Beclin-1 and LC3β immunoreactivities were significantly higher than those in the other groups ($p<0.05$) (Figures 2, 3). p62 immunoreactivity was observed in the nuclei cytoplasm of the tubular epithelial cells. Although the lowest immunoreactivity was observed in the 25 mg/kg ATX + I/R group, the difference was not statistically significant (Figure 4). The immunohistochemical analysis findings are shown in Table 3.

DISCUSSION

In our study, the effect of ATX pretreatment on autophagy was investigated in I/R rats. It was determined that I/R induced oxidative damage in the kidneys. It was observed that ATX administration protected the kidneys against oxidative damage in the groups in which ATX was administered before I/R independent of the dose. However, in the high-dose ATX group, the protection was via autophagy induction.

BUN and creatinine are employed to analyze kidney function, and an increase in BUN and creatinine levels indicates a decrease in kidney functions[21]. The H&E staining method is employed to monitor histopathological changes in kidney tissue. Various studies have reported that ATX administration significantly reduced high BUN and creatinine levels and improved histopathological changes induced by kidney damage[22,23]. Li et al.[24] reported that BUN increased because of kidney damage; however, the change in creatinine was not significant, ATX decreased the high

Table 1. The Kruskal-Wallis test was used to analyze the data, and the Conover test was used for the Kruskal-Wallis test in multiple comparisons. Figures are presented as medians (min-max).

Parameter	Group				
	Sham	I/R	5 mg/kg ATX + I/R	10 mg/kg ATX + I/R	25 mg/kg ATX + I/R
SOD (U/mg)	14.49 (9.57-20.65)	7.75 (5.12-9.92)[a]	13.99 (12.14-16.73)	11.87 (7.67-24.69)	13.75 (9.41-18.61)
MDA (µmol/g tissue)	1.70 (1.47-3.130)	5.66 (3.10-12.14)[b]	2.45 (1.40-4.21)	2.35 (1.33-3.32)	2.01 (1.14-2.81)
TAS (µmol trolox equivalent /L)	11.12 (8.72-13.35)	8.40 (5.73-11.23)[a]	12.60 (8.15-15.81)	13.37 (8.50-17.97)	12.45 (9.81-15.06)
TOS (µmol H_2O_2 equivalent/L)	2.21 (1.96-3.27)	4.54 (3.08-5.57)[b]	2.75 (2.03-3.62)	3.00 (1.76-3.83)	2.91 (2.02-3.98)
BUN (mg/dL)	5.04 (4.10-5.45)	5.59 (5.05-6.99)[b]	4.61 (3.75-5.88)	4.59 (3.84-5.21)	4.77 (4.31-5.74)
Creatinine (mg/dL)	69.51 (54.16-81.18)	78.28 (71.93-91.77)[b]	67.69 (36.86-93.15)	72.20 (28.15-97.96)	54.04 (41.13-73.88)

[a]Significant decrease compared with other groups ($p<0.05$), [b]Significant increase compared with other groups ($p<0.05$). ATX: Astaxanthin, I/R: Ischemia-reperfusion, SOD: Superoxide dismutase, MDA: Malondialdehyde, TAS: Total antioxidant status, TOS: Total oxidant status, min-max: Minimum-maximum, BUN: Blood urea nitrogen

BUN level, and the pathological changes induced by kidney damage were reduced after ATX administration. In the present study, it was determined that BUN and creatinine increased due to I/R-induced kidney damage, and these values approached normal in the ATX + I/R groups, independent of the administration dose.

As reported by previous studies, the antioxidant properties of ATX neutralize free radicals and protect cell membranes, cells, and tissues against oxidative damage[25]. In the current study, the common biomarkers SOD, MDA, TAS, and TOS were employed to analyze I/R-induced oxidative damage. Rats pretreated with ATX exhibited lower MDA and TOS and higher SOD and TAS levels when compared with the I/R group rats, consistent with previous study findings, indicating a strong antioxidant effect of ATX and its protective effect against oxidative damage[22,25-28].

Autophagy plays a critical role in the maintenance of cellular homeostasis under stress conditions. The protective mechanisms associated with autophagy induction have been emphasized in various experimental kidney damage models[29,30]. While it seems reasonable to induce autophagy to protect kidney tissue based on current evidence, other studies have reported that increased autophagy augmented kidney damage[31,32]. Thus,

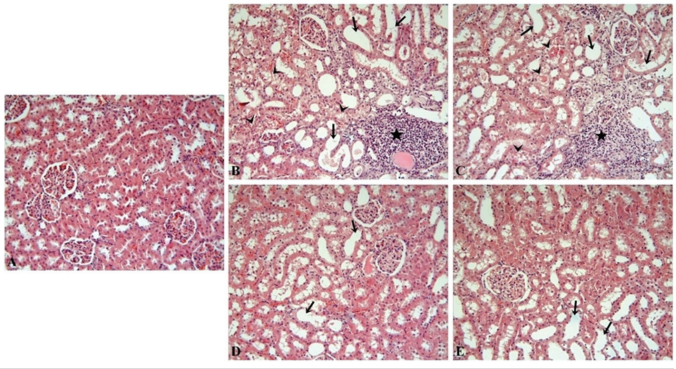

Figure 1. Histopathological findings.

[In the sham group (A), the kidney cortical tissue has a normal histological appearance. In the I/R group (B), necrotic (arrowheads) and dilated (arrows) tubules and inflammatory cell infiltration (star) were noted in the interstitial region. In the 5 mg/kg ATX + I/R group (C), necrotic tubules (arrowheads), dilated tubules (arrows), and inflammatory cell infiltration (star) were observed, similar to the I/R group. In the 10 mg/kg ATX +I/R (D) and 25 mg/kg ATX + I/R (E) groups, significant alleviation in histopathological changes was noted, except for tubular dilatation (arrows)].

ATX: Astaxanthin, I/R: Ischemia-reperfusio

Table 2. Histopathological analysis findings.

Group	Tubular degeneration	Infiltration
Sham	0.0 (0.0-1.0)	0.0 (0.0-0.0)
I/R	2.0 (0.0-3.0)[a]	0.0 (0.0-3.0)[a]
5 mg/kg ATX + I/R	1.0 (0.0-3.0)	0.0 (0.0-3.0)[a]
10 mg/kg ATX + I/R	1.0 (0.0-3.0)[b]	0.0 (0.0-2.0)[b]
25 mg/kg ATX + I/R	1.0 (0.0-3.0)[b]	0.0 (0.0-2.0)[b,c]

[a]Significant increase compared with the sham group ($p<0.0001$). [b]Significant decrease compared with the I/R group ($p<0.0001$). [c]Significant decrease compared with the 5 mg/kg ATX +I/R group ($p=0.0180$). ATX: Astaxanthin, I/R: Ischemia-reperfusion

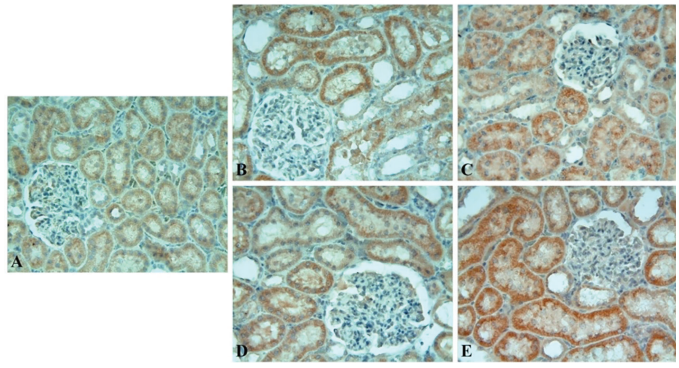

Figure 2. Beclin-1 immunreactivity.

(A: sham group, B: I/R group, C: 5 mg/kg ATX + I/R group, D: 10 mg/kg ATX + I/R group, E: 25 mg/kg ATX + I/R group. Brownish tubule Beclin-1 immunoreactivity in epithelial cells. In the 25 mg/kg ATX + I/R group, immunoreactivity intensity was higher than that in the other groups. Beclin-1 immunoreactivity, 40).

ATX: Astaxanthin, I/R: Ischemia-reperfusion

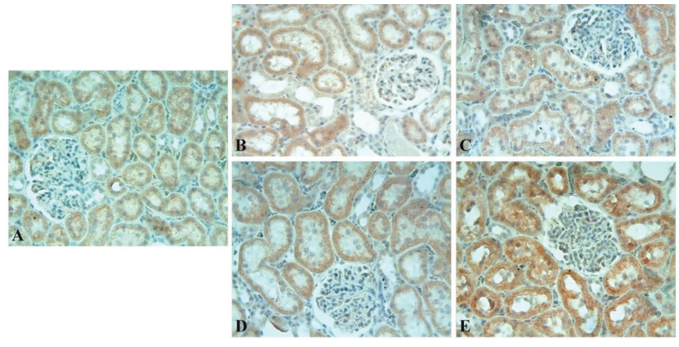

Figure 3. LC3b immunreactivity.

(A: sham group, B: I/R group, C: 5 mg/kg ATX + I/R group, D: 10 mg/kg ATX + I/R group, E: 25 mg/kg ATX + I/R group. Brownish tubule LC3b immunoreactivity in epithelial cells. Immunoreactivity intensity was higher in the 25 mg/kg ATX + I/R group than in the other groups. LC3b immunoreactivity, 40).

there are contradicting views on the protective properties of autophagy in tissues and whether it contributes to I/R induced damages[29,31,32]. ATX also protects tissues against damage via autophagy upregulation. In an experimental study, ATX had protective effects against pancreatic tissue damage by inhibiting autophagy in rats with acute pancreatitis[33]. An experimental study by Shen et al.[34] Reported that autophagy induced liver fibrosis, and ATX had a protective effect on liver tissue via the inhibition of autophagy. Autophagy increased due to dysregulated energy metabolism after I/R in the liver, and ATX reduced the damage caused by autophagy-induced cell necrosis[35]. When these results were evaluated together, ATX protected tissues by inhibiting autophagy. Lee et al.[36] reported that ATX induced autophagy in gastric adenocarcinoma cells induced by *Helicobacter pylori* and protected gastric tissues. In another study, it was reported that ATX augmented autophagy after spinal cord injury and reduced histopathological changes and neuronal degeneration in spinal tissue[37]. It has also been reported in an experimental study that ATX delays the decline in learning and memory ability caused by aging by alleviating cell damage in the brain, i.e., ATX delays age-related cognitive decline by inducing autophagy[38]. These results suggest that ATX protects tissues against damage by inducing autophagy. In our study, ATX protected tissues against damage by inducing autophagy.

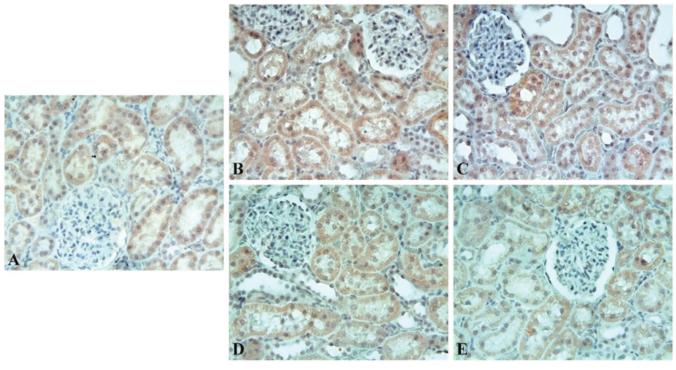

Figure 4. p62 immunoreactivity.

(A: sham group, B: I/R group, C: 5 mg/kg ATX + I/R group, D: 10 mg/kg ATX + I/R group, E: 25 mg/kg ATX + I/R group. Brownish tubule p62 immunoreactivity in epithelial cells. Immunoreactivity intensity was milder in the 25 mg/kg ATX + I/R group than in the other groups. p62 immunoreactivity, 40).

ATX: Astaxanthin, I/R: Ischemia-reperfusion

In our study, histological and biochemical analyses were conducted to monitor kidney tissue damage, and Beclin-1, LC3b and p62 immunoreactivities were analyzed to determine autophagy. Biochemical findings demonstrated that ATX was effective in preserving kidney function and protecting against oxidative damage. Based on the biochemical data, the effects of ATX administration were dose-independent; however, histological analysis revealed no improvement in the 5 mg/kg ATX + I/R group in parallel with the biochemical parameters. It could be suggested that this was due to the need for longer or higher dose administration for histological improvement. Furthermore, high Beclin-1 and LC3b immunoreactivities and low p62 immunoreactivity demonstrated autophagy activation in the 25 mg/kg ATX + I/R group. The decrease in infiltration in the 25 mg/kg ATX + I/R group demonstrated that autophagy induced in the 25 mg/kg ATX + I/R group also protected the tissues against damage.

CONCLUSION

In conclusion, ATX had a protective effect on kidney function and against oxidative damage. Furthermore, high-dose ATX administration protected kidney tissue via autophagy induction in this study.

Table 3. Immunohistochemical analysis findings.

Group	Beclin-1	LC3β	p62
Sham	8.0 (4.0-12.0)	8.0 (4.0-12.0)	4.0 (0.0-12.0)
I/R	8.0 (4.0-12.0)	8.0 (4.0-12.0)	4.0 (0.0-12.0)
5 mg/kg ATX + I/R	8.0 (4.0-12.0)	8.0 (4.0-12.0)	4.0 (0.0-12.0)
10 mg/kg ATX + I/R	8.0 (4.0-12.0)	8.0 (4.0-12.0)	4.0 (0.0-12.0)
25 mg/kg ATX + I/R	12.0 (4.0-12.0)[a]	8.5 (4.0-12.0)[b]	4.0 (0.0-9.0)

[a]Significant increase compared with Sham, I/R, 5 mg/kg ATX + I/R, 10 mg/kg ATX + I/R, and 25 mg/kg ATX + I/R groups ($p<0.05$). [b]Significant increase compared with I/R, 5 mg/kg ATX + I/R, 10 mg/kg ATX + I/R, and 25 mg/kg ATX + I/R groups ($p<0.05$). ATX: Astaxanthin, I/R: Ischemia-reperfusion

Ethics

Ethics Committee Approval: The experimental protocol was examined and approved by the Animal Experiments Local Ethics Committee of Inonu University Faculty of Medicine (protocol no: 2019/A-48) on 23.10.2019.

Informed Consent: Animal experiment study.

Author Contributions

Surgical and Medical Practices: A.K., E.K., N.Y., K.T., U.Y., Concept: A.K., E.K., N.Y., D.O., Design: A.K., E.K., D.O., Data Collection and/or Processing: A.K., E.K., N.Y., K.T., A.Y., U.Y., R.H.C., Analysis and/or Interpretation: A.K., E.K., N.Y., K.T., A.Y., U.Y., R.H.C., Literature Search: A.K., E.K., Writing: A.K., E.K.

Conflict of Interest: The authors have no conflict of interest to declare.

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