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ORIGINAL RESEARCH

# Protective effect of astaxanthin on testis torsion/detorsion injury through modulation of autophagy

Nesibe Yilmaz[1,*], Azibe Yildiz[2], Kevser Tanbek[3], Aysegul Kisaoglu[4], Umit Yilmaz[5], Evren Kose[4]

[1]Department of Anatomy, Faculty of Medicine, Karabuk University, 78050 Karabuk, Turkey
[2]Department of Histology and Embryology, Faculty of Medicine, Inonu University, 44280 Malatya, Turkey
[3]Department of Physiology, Faculty of Medicine, Inonu University, 44280 Malatya, Turkey
[4]Department of Anatomy, Faculty of Medicine, Inonu University, 44280 Malatya, Turkey
[5]Department of Physiology, Faculty of Medicine, Karabuk University, 78050 Karabuk, Turkey

***Correspondence**
nesibeyilmaz@karabuk.edu.tr
(Nesibe Yilmaz)

## Abstract

A significant clinical condition known as testicular torsion leads to permanent ischemic damage to the testicular tissue and consequent loss of function in the testicles. In this study, it was aimed to evaluate the protective effects of Astaxanthin (ASTX) on testicular damage in rats with testicular torsion/detorsion in the light of biochemical and histopathological data. Spraque Dawley rats of 21 were randomly divided into three groups; sham, testicular torsion/detorsion (TTD) and astaxanthin + testicular torsion/detorsion (ASTX + TTD). TTD and ASTX + TTD groups underwent testicular torsion for 2 hours and then detorsion for 4 hours. Rats in the ASTX + TTD group were given 1 mg/kg/day astaxanthin by oral gavage for 7 days before torsion. Following the detorsion process, oxidative stress parameters and histopathological changes in testicular tissue were evaluated. Malondialdehyde (MDA) and total oxidant status (TOS) levels were significantly decreased in the ASTX group compared to the TTD group, while superoxide dismutase (SOD), glutathione (GSH) and total antioxidant status (TAS) levels were increased ($p < 0.05$). Moreover, histopathological changes were significantly reduced in the group given ASTX ($p < 0.0001$). It was determined that ASTX administration increased Beclin-1 immunoreactivity in ischemic testicular tissue, while decreasing caspase-3 immunoreactivity ($p < 0.0001$). Our study is the first to investigate the antiautophagic and antiapoptotic properties of astaxanthin after testicular torsion/detorsion based on the close relationship of Beclin-1 and caspase-3 in ischemic tissues. Our results clearly demonstrate the protective effects of ASTX against ischemic damage in testicular tissue. In ischemic testicular tissue, ASTX contributes to the survival of cells by inducing autophagy and inhibiting the apoptosis.

## Keywords

Testis; Torsion; Astaxanthin; Oxidative stress; Beclin-1; Caspase-3


  

## Efecto protector de la astaxantina en la lesión por torsión/detorsión testicular a través de la modulación de la autofagia


### Resumen

Una condición clínica significativa conocida como torsión testicular conduce a daño isquémico permanente al tejido testicular y consecuente pérdida de función en los testículos. En este estudio, el objetivo fue evaluar los efectos protectores de la astaxantina (ASTX) sobre el daño testicular en ratas con torsión/detorsión testicular a la luz de los datos bioquímicos e histopatológicos. Se dividieron al azar ratas Spraque Dawley de 21 en tres grupos; simulación, torsión/detorsión testicular (TTD) y astaxantina+torsión/detorsión testicular (ASTX + TTD). Los grupos TTD y ASTX + TTD se sometieron a torsión testicular durante 2 horas y luego a detorsión durante 4 horas. Las ratas del grupo ASTX + TTD recibieron 1 mg/kg/día de astaxantina por sonda oral durante 7 días antes de la torsión. Tras el proceso de detorsión, se evaluaron los parámetros de estrés oxidativo y los cambios histopatológicos en el tejido testicular. los niveles de malondialdehído (MDA) y estado oxidante total (TOS) disminuyeron significativamente en el grupo ASTX en comparación con el grupo TTD, mientras que los niveles de superóxido dismutasa (SOD), glutatión (GSH) y estado antioxidante total (TAS) aumentaron ($p < 0.05$). Además, los cambios histopatológicos se redujeron significativamente en el grupo que recibió ASTX ($p < 0.0001$). Se determinó que la administración de ASTX aumentó la inmunorreactividad de Beclin-1 en tejido testicular isquémico, mientras que disminuyó la inmunorreactividad de caspasa-3 ($p < 0.0001$). Nuestro estudio es el primero en investigar las propiedades antiautofágicas y antiapoptóticas de la astaxantina después de una torsión/detorsión testicular basada en la estrecha relación de Beclin-1 y caspasa-3 en tejidos isquémicos. Nuestros resultados demuestran claramente los efectos protectores de ASTX contra el daño isquémico en el tejido testicular. En el tejido testicular isquémico, ASTX contribuye a la supervivencia de las células al inducir la autofagia e inhibir la apoptosis.



### Palabras Clave

Testículo; Torsión; Astaxantina; Estrés oxidativo; Beclin-1; Caspasa-3


## 1. Introduction

Testicular torsion is an acute urological condition that can cause permanent ischemic damage to the testis because of the deterioration of blood supply to the testis due to the rotation of the spermatic cord around itself. If the testicular torsion is not intervened promptly, necrosis may develop and require urgent surgery. Consequentially, if neglected may lead to loss of testicular function and infertility [1]. The extent is just as prominent as the continuance of torsion in terms of damage. As the degree of torsion increases, a complete blockage occurs in the arteries in the spermatic cord, and thus, ischemic damage occurs more rapidly [2]. 720° torsion must occur in order for the blood flow of the arteries that provide blood flow to the testicle to be completely disrupted and for complete necrosis of the testicular tissue to occur. In most cases, torsions of 360–720° are observed. In 360° testicular torsion, treatments applied within the first 6 hours allow most of the testicular tissue to be saved, while treatments performed after 24 hours may result in the loss of the entire testicle [3]. To eliminate ischemic damage, blood flow must be restored. However, reperfusion contributes to the increase in free oxygen radicals, causing even more damage to the tissue [4].

Antioxidants are important molecules that are a part of the body's line of defense against the potential harm of free oxygen radicals formed during oxidative stress [5]. Astaxanthin (ASTX) is a molecule in the xanthophyll subclass of carotenoids with strong antioxidant capacity. Astaxanthin differs from other carotenoids in that each of its ring structures has two additional oxygenated groups, strengthening its antioxidant capacity [6]. It has been shown that its antioxidant capacity is approximately 100 fold stronger than vitamin E, which is a well-known potent antioxidant [7]. In addition, it has recently attracted significant attention with its DNA repair, cell regeneration, neuroprotective, anti-inflammatory, anti-diabetic, anticancer, anti-apoptotic and anti-autophagic properties [8–10]. Studies indicate that a healthy diet containing high amounts of antioxidants may be effective in increasing semen quality and fertility. Researchers have demonstrated the positive effects of astaxanthin on sperm quality and fertility by significantly reducing the secretion of reactive oxygen species and inhibin B by Sertoli cell [8]. Reveal of its many positive effects on human health has made astaxanthin a nutritional supplement in the daily diet of individuals [11].

Two recent studies demonstrated the benefits of astaxanthin on testicular ischemia/reperfusion. In these studies, the protective effects of a single dose of astaxanthin administered during or after detorsion on testicular tissue and its protective effects against oxidative damage and histopathological changes in testicular torsion were investigated [4, 12]. Although astaxanthin has been shown to play a protective role against testicular ischemia/reperfusion injury, related metabolic pathways are still unknown.

In this study, the antioxidative, antiapoptotic and antiautophagic properties of astaxanthin as protection from testicular ischemia/reperfusion injury were investigated. Moreover, the possible protective effects of astaxanthin usage prior to ischemic damage to testicular tissue against ischemia/reperfusion injury were evaluated. The mechanisms of apoptosis and autophagy, two cell death pathways functioning in coordination in ischemic tissues, were considered and it was aimed to reveal *via* which pathway astaxanthin shows its protective effect on testicular tissue against ischemic damage.

## 2. Material and methods

### 2.1 Animal care

Throughout the experiments, rats were housed in 21 ± 1 ℃ and a light/dark period of 12 hours, normal tap water and standard rat chow were provided ad libitum. All applications were conducted at Inonu University Experimental Animal Application and Research Center.

### 2.2 Study design

Spraque Dawley rats of 21 weighing 250–300 g were included. Power analysis showed that in the case of a quantity of type I error (alpha) of 0.05, test's power (1 − beta) of 0.8, effect size of 0.83, and number of groups of 3, the least number of subjects needed to determine a statistical significance should be 7. Rats with similar body weights were separated into 3 groups (n = 7), which were weighed prior to the study.

Sham group: Rats in this group were given 1 mL ASTX solvent (olive oil) by oral gavage for 7 days. With the conclusion of the 7th day, sham surgery was performed on the animals in this group. The left testis was reached with a vertical incision made from the area above the scrotum. The left testis was reached with a vertical incision made from the area above the scrotum. The testis was rapidly returned to its normal state and the skin was sutured.

Testicular torsion/detorsion (TTD) group: Rats in this group were given 1 mL of ASTX solvent (olive oil) by oral gavage for 7 days. With the conclusion of the 7th day, rats in this group underwent testicular torsion/detorsion surgery. The left testicle of the rats was accessed through a left scrotal incision. The testicle was rotated 720° clockwise and fixed to the scrotum wall with 4/0 silk suture. The incision site was closed, 2 hours of torsion followed by 24 hours of reperfusion [13].

Astaxanthin+testicular torsion/detorsion (ASTX + TTD) group: Rats in this group recieved 1 mL ASTX (1 mg/kg) (Galenik, Cas no: 742-61-7, İzmir, Turkey) by oral gavage for 7 days [14]. With the conclusion of the 7th day, they underwent testicular torsion/detorsion surgery. The left testicle of the rats was accessed through a left scrotal incision. The testicle was rotated 720° clockwise and fixed to the scrotum wall with 4/0 silk suture. The incision site was closed, 2 hours of torsion followed by 24 hours of reperfusion [13].

### 2.3 Surgical procedure

Anesthesia was achieved with 70 mg/kg of ketamine and 8 mg/kg of xylazine given intramuscularly. Anesthesia was adjusted so that subjects were unresponsive to pain and resume spontaneous breathing during the experiment. The left testicle was reached with a left vertical incision made over the scrotum and the testicle was excised together with its vascular structures. Testicular torsion was generated with 720° clockwise rotation of the left testis, and fixation was achieved with 4/0 silk sutures to prevent detorsion and the scrotum was closed with 4/0 silk sutures. Sutures were opened 2 hours after the procedure and detorsion was achieved for 4 hours [13, 15]. Following detorsion, the rats underwent orchiectomy, tissue samples were taken and the rats were sacrificed. Testicular tissues were divided into two equal parts along its vertical axis. One of the pieces was used for biochemical analysis and the other for histological analysis.

### 2.4 Biochemical analyses

Testicular tissues were stored at −80 °C until studied and transferred to +4 °C overnight a day before. On the examination day, the wet tissue weights of each were weighed and taken into glass tubes, and cold Tris-HCl buffer (pH 7.4, 0.2 mM) was added 10 fold the wet tissue weight, and homogenized in a homogenizer at 16000 rpm for 3 minutes. Malondialdehyde (MDA) levels were established from the obtained homogenates. A part of the homogenates were centrifuged at 2200g for 1 hour at +4 °C and supernatants were collected, which were used for the determination of superoxide dismutase (SOD) and glutathione (GSH) levels, and total antioxidant capacity (TAS) and total oxidant capacity (TOS). It is known that free oxygen radicals that occur following ischemia/reperfusion injury cause oxidation of membrane lipids and accumulation of MDA, a toxic substance, is observed in tissues as a result of lipid oxidation. The differences in MDA levels between the groups were evaluated to determine the extent of oxidative damage. Enzymatic antioxidants, namely SOD and GSH, are components of the defense against damage by free radicals in tissues, which were also evaluated [16].

MDA activities, a lipid peroxidation marker, were determined by the thiobarbituric acid reaction method and given as nmol/g [17].

SOD enzyme activities were determined by the nitro blue tetrazolium (NBT) reduction method and expressed as U/mg protein [18].

GSH concentrations were measured as previously described and expressed as $\mu$mol/g protein [19].

Total Antioxidant Capacity (TAS) and total oxidant capacity (TOS) measurements were performed using the commercial TAS kit (Cat no: 201-11-2672, SunRed Biotechnology, Shanghai, China) and TOS kit (Cat no: 201-11-1669, SunRed Biotechnology, Shanghai, China) on an immunoplate reader with Biotek HT Snynergy Gen 5 software (Biotek, Winooski, VT, USA). TAS levels were given as $\mu$mol Trolox Equivalent/L, and TOS levels as $\mu$mol $H_2O_2$ equivalent/L.

### 2.5 Histopathological analysis

The testes were fixed in 10% neutral formaldehyde. After fixation, specimens were dehydrated in 70%, 80%, 90%, 96% and 99% alcohol series. Then, they were cleared in xylene, and embedded in paraffin and stained with haematoxylin—eosin (H&E).

In each study group, at least 100 random seminiferous tubules were examined in terms of degenerative changes such as atrophy, germ-cell degeneration, meiotic arrest, multi-nucleated giant cell formation, and spilling of immature germ cells in the lumen. Tubular degeneration was scored as previously described [20]. Additionally, diameter and epithelium thickness of at least 100 random seminiferous tubules in each study group were measured. All assessments and measurements were performed with a Leica DFC 280

optical microscope and the Leica Q Win analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

### 2.6 Immunohistochemial analysis

For immunohistochemical analysis, 4 $\mu$m thick tissue sections were deparaffinized, rehydrated and in order to detect their levels, beclin-1 (ab62557, Abcam) and caspase-3 (ab49822, Abcam) primer antibodies and biotinylated secondary antibodies were utilized as previously described [21]. Samples were visualized with the chromogenic substrates 3-amino-9-ethylcarbazole (AEC), counterstained with hematoxylin, mounted in glass slide.

Immunohistochemical examinations demonstrated the presence of beclin-1 and caspase-3 immunostaining in the germinal cells of the seminiferous tubules. For immunohistochemical evaluation, in each study group, at least 100 random seminiferous tubules were examined, and H scorings were performed. The H scores were determined by multiplying the prevalence and intensity of the immunostaining. Then, sections were graded as described previously [22]. Leica DFC 280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK) was used for immunohistochemical evaluations and measurements.

### 2.7 Statistical analysis

Findings were studied using the IBM SPSS software program for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA) and presented as either median (minimum–maximum) or means ± standard deviation (SD) contingent upon the overall variable distribution. The normality of the distribution was verified utilizing the Shapiro-Wilk test. Normally distributed data were studied by one-way analysis of variance (ANOVA) followed by the Tamhane *post hoc* test, while non-normally distributed were compared by the Kruskal-Wallis H test. Significant differences were, compared utilizing the Mann-Whitney U test with Bonfernoni correction. The outcome was accepted significant if $p < 0.05$.

## 3. Results

### 3.1 Effects of ASTX on oxidative stress in testes tissue

It was observed that ASTX administration significantly increased SOD and GSH levels in ischemic tissue, while decreasing MDA levels (Fig. 1) ($p < 0.05$). Thus, it was revealed that ASTX protects tissues from oxidative damage in testicular ischemia.

When TAS and TOS levels were evaluated between the groups, it was determined that ASTX administration significantly elevated the TAS level compared to the TTD group, while it decreased the TOS level (Fig. 2) ($p < 0.05$).

### 3.2 Histopathological findings

The cross sections of testes in the sham group were intact and seminiferous tubule epithelia were organized in concentric layers containing Sertoli cells in close contact with differentiating germ cells. Spermatogenic cells at various stage of division were observed in the seminiferous epithelia. The lumens of seminiferous tubules were plenteous with spermatid and sperm (Fig. 3).

However, obvious degenerative changes in seminiferous tubules were seen in the TTD group. Seminiferous tubule structures were shrunken and separated from each other in this group. The seminiferous epithelia of testes in the TTD group exhibited germ-cell degeneration, meiotic arrest, multinucleated giant cell formation and spilling of immature germ cells in the lumen. The difference between TTD and sham groups regarding histopathological changes was statistically significant ($p < 0.0001$). Moreover, the mean seminiferous epithelium thickness (SET) and seminiferous epithelium diameter (STD) were statistically decreased in the TTD in comparison to the sham ($p < 0.0001$).

Conversely, histopathological changes were markedly reduced in the ASTX + TTD in comparison to the TTD ($p < 0.0001$) although slight degenerative changes such as meiotic arrest, spilling of immature germ cells in the lumen continued. Moreover, ASTX administration was increased the mean SET and STD ($p < 0.0001$). The histopathological scores, mean SET and STD of each group are given in Table 1.

**TABLE 1. The histopathological scores, seminiferous epithelium thickness and seminiferous tubule diameter for each group.**

| Groups | HS Med (min–max) | SET Mean ± SD | STD Mean ± SD |
|---|---|---|---|
| Sham | 0 (0–1) | 51.4 ± 8.3 | 292.3 ± 23.2 |
| TTD | 2 (0–3)[a] | 39.7 ± 7.0[c] | 248.7 ± 18.3[c] |
| ASTX + TTD | 0 (0–3)[b] | 50.1 ± 8.1[d] | 261.1 ± 22.0[d] |

[a] *Statistically higher than sham group ($p < 0.0001$).*
[b] *Statistically lower than TTD group ($p < 0.0001$).*
[c] *Statistically lower than sham group ($p < 0.0001$).*
[d] *Statistically higher than TTD group ($p < 0.0001$).*
*TTD: testicular torsion/detorsion; ASTX + TTD: astaxanthin + testicular torsion/detorsion; HS: Histopathologic score; SET: seminiferous epithelium thickness; STD: seminiferous epithelium diameter; SD: standard deviation.*

### 3.3 Immunohistochemical findings

#### 3.3.1 Beclin-1 immunoreactivity

Immunoreactivity for beclin-1 was found in the seminiferous epithelia of testes in all groups. Beclin-1 immunoreactivities were evident especially in the basal regions of the epithelia (Fig. 4). An obvious increase in the immunoreactivity of beclin-1 was observed in the TTD in comparison to the sham ($p < 0.0001$). Conversely, the immunoreactivity obtained in the ASTX + TTD was statistically higher compared to both the sham and TTD groups. The scores of beclin-1 immunoreactivity assesment of each group are given in Table 2.

#### 3.3.2 Caspase-3 immunoreactivity

The sham group revealed a minor caspase-3 immunoreactivity in the seminiferous epithelium (Fig. 5A). However, it was

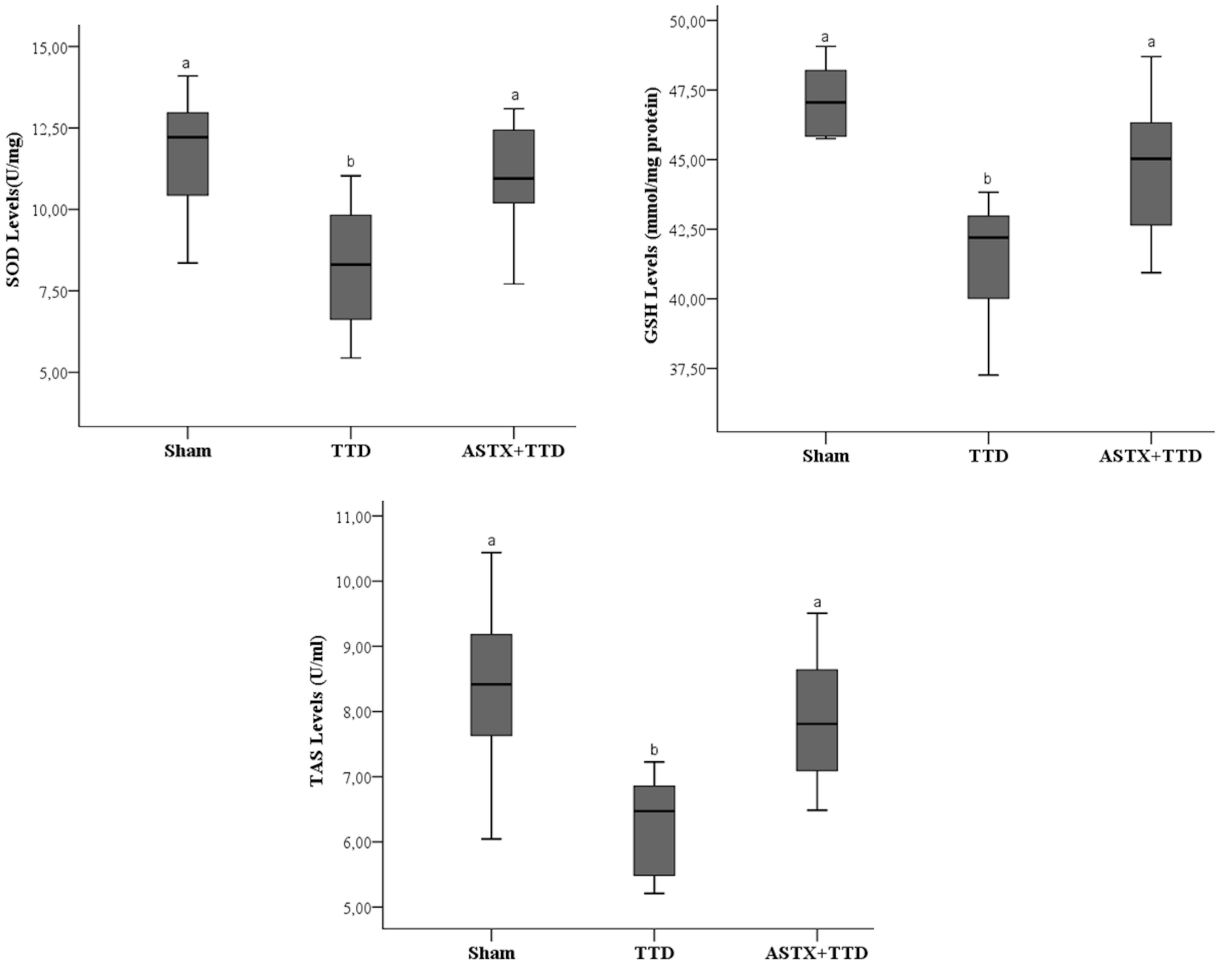


**FIGURE 1. Comparison of SOD, GSH and TAS levels between groups.** (The comparison of the variables between groups performed by the one-way ANOVA. Tamhane's test was used for all parameters in multiple comparisons. Data were given as mean ± SD. Differences between groups were indicated by letters. $p < 0.05$ values considered statistically significant. $^{a}$Statistically higher than TTD group ($p < 0.05$), $^{b}$Statistically lower than sham group ($p < 0.05$)). TTD: testicular torsion/detorsion; ASTX + TTD: astaxanthin + testicular torsion/detorsion; SOD: superoxide dismutase; GSH: glutathione; TAS: total antioxidant status.

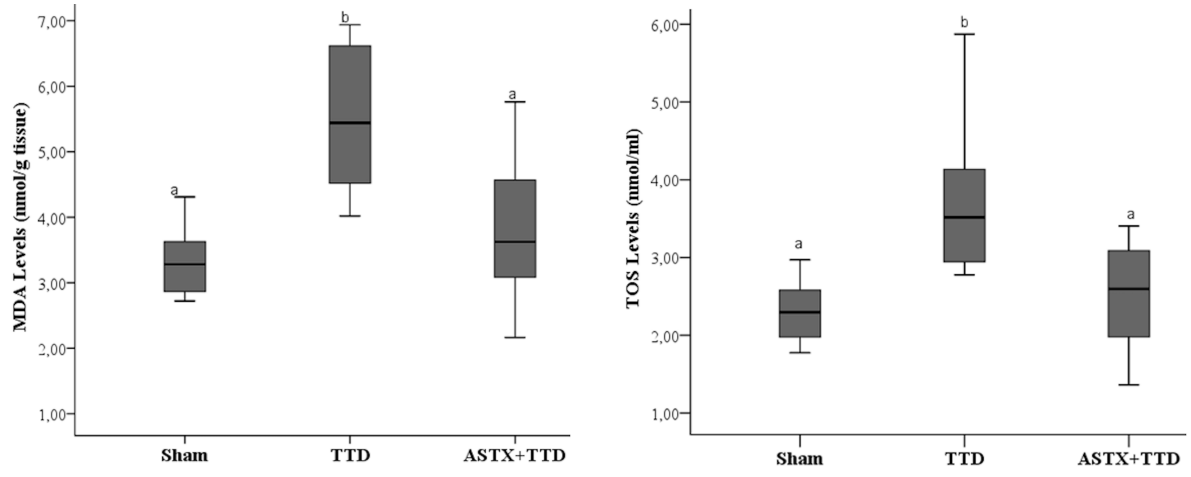


**FIGURE 2. Comparison of MDA and TOS levels between groups.** The comparison of the variables between groups performed by the one-way ANOVA. Tamhane's test was used for all parameters in multiple comparisons. Data were given as mean ± SD. Differences between groups were indicated by letters. $p < 0.05$ values considered statistically significant. $^{a}$Statistically lower than TTD group ($p < 0.05$), $^{b}$Statistically higher than sham group ($p < 0.05$). TTD: testicular torsion/detorsion; ASTX + TTD: astaxanthin + testicular torsion/detorsion; MDA: malondialdehyde; TOS: total oxidant status.

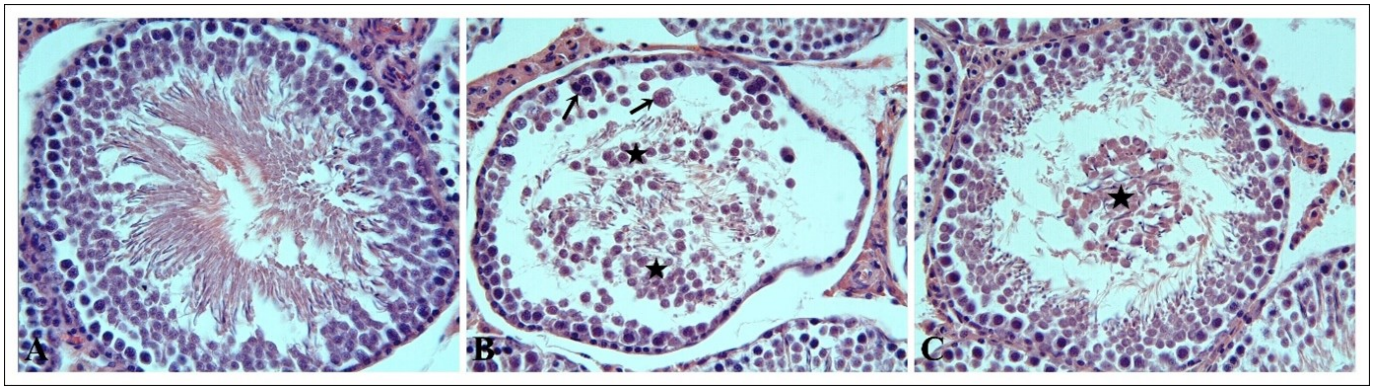


**FIGURE 3. Histopathological changes.** The sham (A), seminiferous tubule is normal histological appearance. The TTD group (B), multi-nucleated giant cell formation in the seminiferous epithelium (arrows) and spilling of immature germ cells in the lumen seminiferous tubule (asteriks) are showed. The ASTX + TTD group (C), it is noteworthy that the histopathological changes are significantly alleviated. H&E staining ×400.

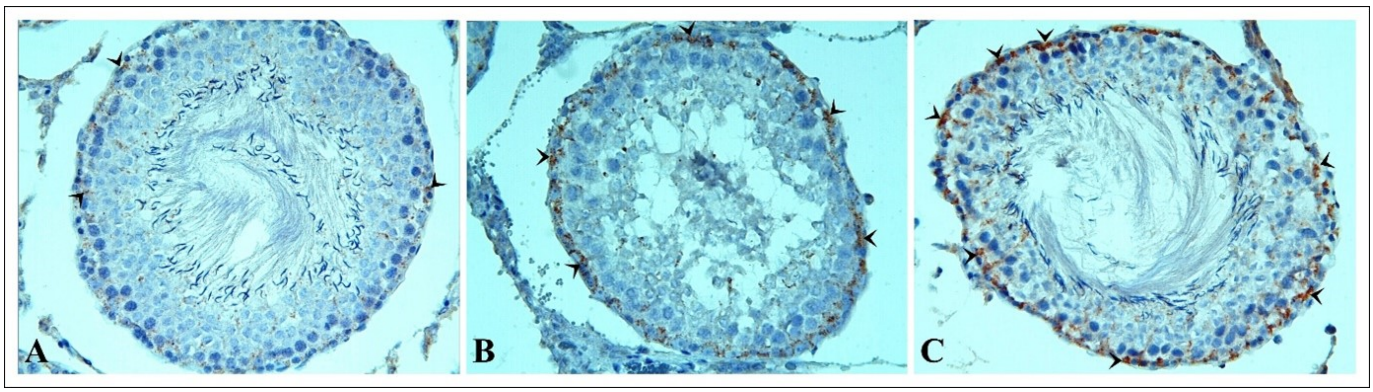


**FIGURE 4. Beclin-1 immunoreactivity.** Beclin-1 immunoreactivity is observed in sham group (A). In TTD group (C), a increase in Beclin-1 immunoreactivity (arrowheads) is noticed. Significantly higher Beclin-1 immunoreactivity is observed in the ASTX + TTD group (C) compared to the sham and TTD groups.

**TABLE 2. The H scores beclin-1 and caspase-3 for each group.**

| Groups | Beclin-1 Med (min–max) | Caspase-3 Med (min–max) |
|---|---|---|
| Sham | 1 (0–4) | 3 (1–6) |
| TTD | 4 (1–9)[a] | 8 (4–12)[a] |
| ASTX + TTD | 6 (4–9)[b] | 6 (4–12) |

[a] *Statistically higher than sham group ($p < 0.0001$).*
[b] *Statistically lower than TTD group ($p = 0.031$).*
*TTD: testicular torsion/detorsion; ASTX + TTD: astaxanthin + testicular torsion/detorsion.*

prominently higher in the TTD in comparison to the sham ($p < 0.0001$) (Fig. 5B). A slight decrease in caspase-3 immunoreactivity was noted in the ASTX + TTD in comparison to the TTD (Fig. 5C), however, it was not statistically significant. The scores of caspase-3 immunoreactivity of each group are given in Table 2.

## 4. Discussion

In the present study, the protective effects of oral ASTX administration before torsion-detorsion were analyzed since ASTX is a food supplement that individuals could use daily. Similar to previous reports, we observed that ASTX significantly reduced testicular ischemia/reperfusion injury. In the ASTX administration group, SOD and GSH levels increased, and MDA level decreased. These findings indicated that ASTX reduced oxidative damage in testicular tissue. Histopathological analysis results also evidenced that ASTX reduced I/R damage in testicular tissue. Histopathological symptoms significantly decreased and mean SET and STD levels increased in the ASTX group when compared to the TTD group. Baskovic *et al.* [4] conducted the first study on the therapeutic effects of ASTX in post-testicular torsion ischemic damage. In the study, they analyzed the effects of a single dose intraperitoneal ASTX administration at the beginning and 45 minutes after testicular detorsion. They conducted a detailed histopathological analysis of the testicular tissue and all morphological parameters in seminiferous tubules (mean seminiferous epithelial thickness, seminiferous tube diameter, tubular area, lumen area, epithelial height, Johnsen score). They reported that the mean seminiferous tubule diameter, epithelial height and Johnsen score were higher in the ASTX administration groups when compared to the torsion group. Thus, they argued that ASTX, a powerful antioxidant, played a therapeutic role in testicular I/R damage [4]. Our histological

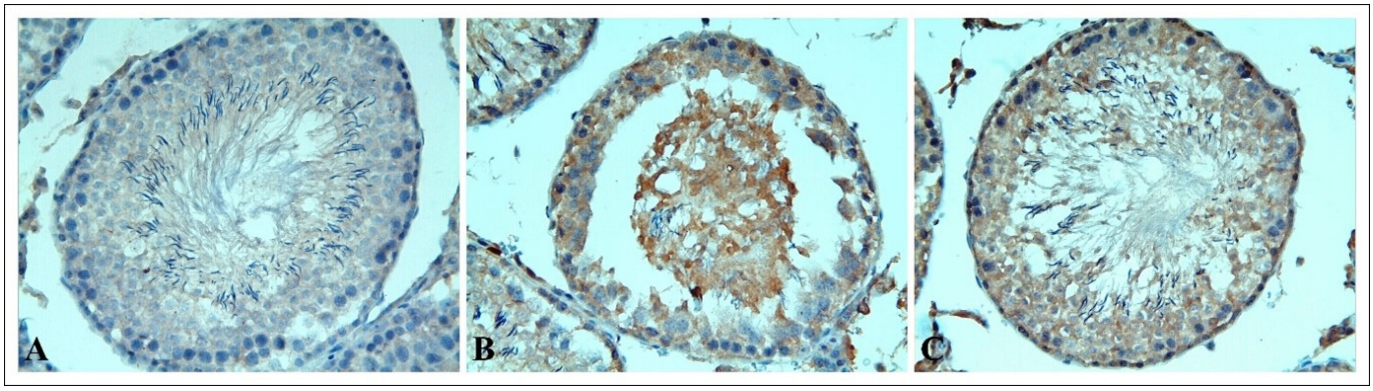


**FIGURE 5. Slight caspase-3 immunoreactivity.** Slight caspase-3 immunoreactivity is observed in sham group (A). In TTD group (C), a increase in caspase-3 immunoreactivity is noticed. On the other hand, a mild decrease in caspase immunoreactivity is showed in the ASTX + TTD group (C) compared with TTD group. Caspase-3 immunostaining ×400.

findings were similar to the study conducted by Baskovic *et al*. [4]. In the present study, it was observed that the histopathological score decreased, and the mean seminiferous epithelial thickness and tubule diameter increased in the ASTX group. Demir *et al*. [23] investigated the effects of ASTX on oxidative stress in testicular I/R injury and reported similar findings. They reported that ASTX administered 30 minutes before detorsion significantly reduced MDA, TOS and oxidative stress index (OSI) levels, and increased TAS and catalase (CAT) levels. Their histopathological analysis revealed that the Johnsen score increased in the ASTX group. Baskovic *et al*. [12] further detailed their morphological studies that revealed the therapeutic role of ASTX in testicular ischemia with biochemical and immunohistochemical analyzes. They reported that intraperitoneal ASTX administration 45 minutes after detorsion decreased MDA levels and increased SOD and glutathione peroxidase (GPx) levels. In the same study, the authors also investigated the effects of ASTX on apoptosis and demonstrated that the number of caspase-3 positive cells decreased in the ASTX administration group. Their study focused on the effects of acute post-testicular I/R injury. Detorsion period was limited to 90 minutes. Previous studies have shown that torsion of at least 1 hours is required for serious damage to the testicular tissue [24–26]. However, in actual clinical cases, the time between the moment of torsion and surgical intervention often exceeds 90 minutes. The authors also argued that it would be beneficial to prolong the torsion period. In the present study, we determined that a torsion of 2 hours would be similar to clinical applications and implemented 2 hours of torsion period. We focused on the protective effect of ASTX rather than its therapeutic effect. It is also known that free oxygen radicals induced by ischemia-reperfusion injury induce apoptosis and autophagy pathways [27]. The present study attempted to determine the effects of ASTX on both apoptosis and autophagy in testicular I/R injury. Caspase-3 plays a key role in apoptosis. It induces DNA fragmentation by activating endonucleases, initiating apoptosis [28]. Autophagy is a lysosomal pathway where misfolded or unfolded proteins, dysfunctional cell components, and toxic cytosolic substances are engulfed by the double membranes. The most important step in the autophagy is the development of autophagosome structures and the fusion of these structures and lysosomes by recognizing the target organelles that would be degraded, and three autophagic proteins, namely Beclin-1, microtubule-associated protein-1 light chain-3 (LC3) and p62, play a key role in this process [28, 29]. Since the autophagy process works in coordination with apoptosis, it is very important to evaluate both pathways together in ischemic processes. Studies have shown that Beclin-1 is the main gene that plays a role in ischemia-induced autophagy and that a decrease in the expression induces the autophagy process [30]. The increased level of the antiapoptotic protein Bcl-2 inhibits apoptosis by increasing the release of cytochrome C from the mitochondrial membrane and decreasing caspase-3 expression [31]. When Rami *et al*. [32] evaluated the expression of Beclin-1 and caspase-3 in ischemic brains, they stated that caspase-3 was not positive in all Beclin-1-expressed cells. Thus, they have suggested that Beclin-1 has a repair function *via* autophagy or that not all dying cells undergo DNA fragmentation or that Beclin-1 may have undefined functions on cerebral ischemia. Antiautophagic and antiapoptotic effects of astaxanthin have also been demonstrated in different studies [33]. Several studies reported that ASTX modulated autophagy [29, 30, 34]. Lee *et al*. [35], demonstrated the neuroprotective effects of ASTX in global cerebral ischemia in rats and suggested that these effects could be associated with the antioxidant properties of ASTX. It was reported that ASTX reduced ischemia-induced damages in ischemic rat brain tissues by inhibiting oxidative stress, reducing glutamate release, and anti-apoptosis. These findings suggested that ASTX could be used clinically to prevent ischemic diseases [36]. The protective and therapeutic effects of ASTX in ischemic damage in ischemic optic neuropathy rats were investigated, and it was reported that ASTX protected visual functions and reduced apoptosis in retinal ganglion cells [37]. Furthermore, it was reported that 14 days of ASTX administration with oral gavage before renal I/R injury prevented I/R injury by significantly reducing the histopathological score and the number of apoptotic cells in kidney tissues [38]. Similarly, it could be suggested that ASTX intake as a daily food supplement could prevent and treat ischemic damage. To date, the effects of ASTX on testicular I/R injury were only investigated by Baskovic *et al*. [4, 12]. However, there is no study where the effects of ASTX on autophagy in testicular I/R injury was investigated. Caspase-3

is a protein that plays a key role in apoptosis. Beclin-1, which plays a significant role in autophagy, allows the development of autophagosomes, and includes two caspase-3 binding sites. When Caspase-3 binds to Beclin-1, Beclin-1 is destructed. Thus, the cell turns to the apoptosis pathway instead of autophagy [28]. Thus, it is important to analyze both pathways. In the presented study, we investigated the antiautophagic and antiapoptotic properties of astaxanthin following testicular torsion/detorsion based on the close relationship of Beclin-1 and caspase-3 in ischemic tissues. Beclin-1 immunoreactivity was decreased in subjects given astaxanthin, which indicates induction of autophagy contributed to the survival of the cells in testicular tissue. It was also determined that astaxanthin administration reduced caspase-3 immunoreactivity and suppressed apoptosis in testicular tissue.

## 5. Conclusions

It is known that autophagy and apoptosis processes are very important in testicular ischemia/reperfusion injury. Regarding our findings, astaxanthin greatly reduces ischemic damage in testicular tissue by increasing the autophagy pathway and suppressing apoptosis. Furthermore, it protects tissues from oxidative damage by minimizing free radicals that occur after ischemia/reperfusion.

## 6. Limitations of the study

This study is the first to investigate the effects of ASTX on autophagy in testicular I/R injury. Thus, Beclin-1 immunoreactivity, an important autophagic gene, was analyzed. In addition to Beclin-1, LC3 and p62 are essential proteins that play a role in autophagy. To further elucidate the effects of ASTX on autophagy, we plan to analyze the immunoreactivity of these two proteins in a future study.

ASTX was administered orally for 7 days before ischemia. Based on the recent reports in the literature, the most adequate dose and administration period for daily oral ASTX intake will be redetermined. The planned future study would aim to better determine the possible effects of ASTX.

The present study findings are promising in the sense that ASTX could be an effective clinical agent. However, these effects should be elucidated with further detailed analyses. Nevertheless, the present study could provide a solid foundation for future studies.

### AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### AUTHOR CONTRIBUTIONS

NY and UY—study design, data collection and analyses; NY, KT, UY and AK—experimental surgery; KT, AK and NY—biochemical examination; AY—histochemical examination; NY, UY and EK—manuscript writing. All authors have read and approved the final version of the study and acknowledge that they are responsible for all aspects of the study.

### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was carried out with the authorization of the Animal Experiments Local Ethics Committee of Faculty of Medicine, Inonu University (Protocol no: 2020/4-2). All applications were performed as stated in the Ethics Committee's guidelines.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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