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Protective effects of antithrombin III on retinal ischemia/reperfusion injury in rats: A histopathologic study

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PURPOSE. To investigate the effect of antithrombin III (AT III) on retinal ischemia/reperfusion (I/R) injury in rats.
METHODS. The study was carried out on 10 Wistar albino rats (20 eyes) and four-vessel occlusion method was employed to induce retinal ischemia in this study. Rats were divided into two groups: Group I (control group, 10 eyes) and Group II (AT III, 10 eyes). In both groups, vertebral arteries were occluded bilaterally an electric needle coagulator under an operating microscope. A total of 48 hours after the initial procedure, the rats were re-anesthetized and both common carotid arteries were clamped to interrupt blood flow. In Group II, rats were injected intravenously with 250 U/kg of AT III 5 minutes before the induction of ischemia. Duration of ischemia was 30 minutes. At the end of this period, clamp was removed for the reperfusion of the eye for 4 hours. Following the reperfusion period, the animals were killed by decapitation. Retinal sections were evaluated under light and electron microscope. The signs of I/R injury at the microscopic level, i.e., cellular degeneration, vacuolization between retinal layers, increase in the retinal thickness due to edema, mononuclear cell infiltration, and apoptotic cells, were recorded for each group.
RESULTS. Retinal sections obtained from the rats in the AT III group revealed a well preserved retinal structure. When average thickness values of the two groups were compared to each other, the difference was significant with respect to inner nuclear and inner plexiform layers indicating increased retinal thickness values in Group I due to tissue edema resulting from I/R injury. Similarly, mononuclear cell infiltration and apoptotic cell counts were found to be significantly higher in control group compared to AT III group showing the inhibitory effect of AT III on leukocyte infiltration and apoptotic cell death in rat retina.
CONCLUSIONS. Antithrombin III attenuated I/R injury in rat retina. (Eur J Ophthalmol 2005; 15: 267-73)

KEY WORDS. Antithrombin III, Apoptosis, Ischemia/reperfusion injury, Retinal ischemia, Thrombin

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INTRODUCTION

Ischemia is thought to contribute to the pathophysiology of several ophthalmic disorders, including glaucoma, retinal vascular occlusions, and diabetic retinopathy. Inadequate blood supply leads to tissue hypoxia, depletion of energy-rich phosphates, accumulation of metabolic waste products including reactive oxygen species, and cellular edema, all of which may cause cellular injury (1, 2). Reinstitution of blood flow is essential to prevent irreversible ischemic cellular damage. However, reperfusion itself may cause additional tissue injury. The phenomenon in which

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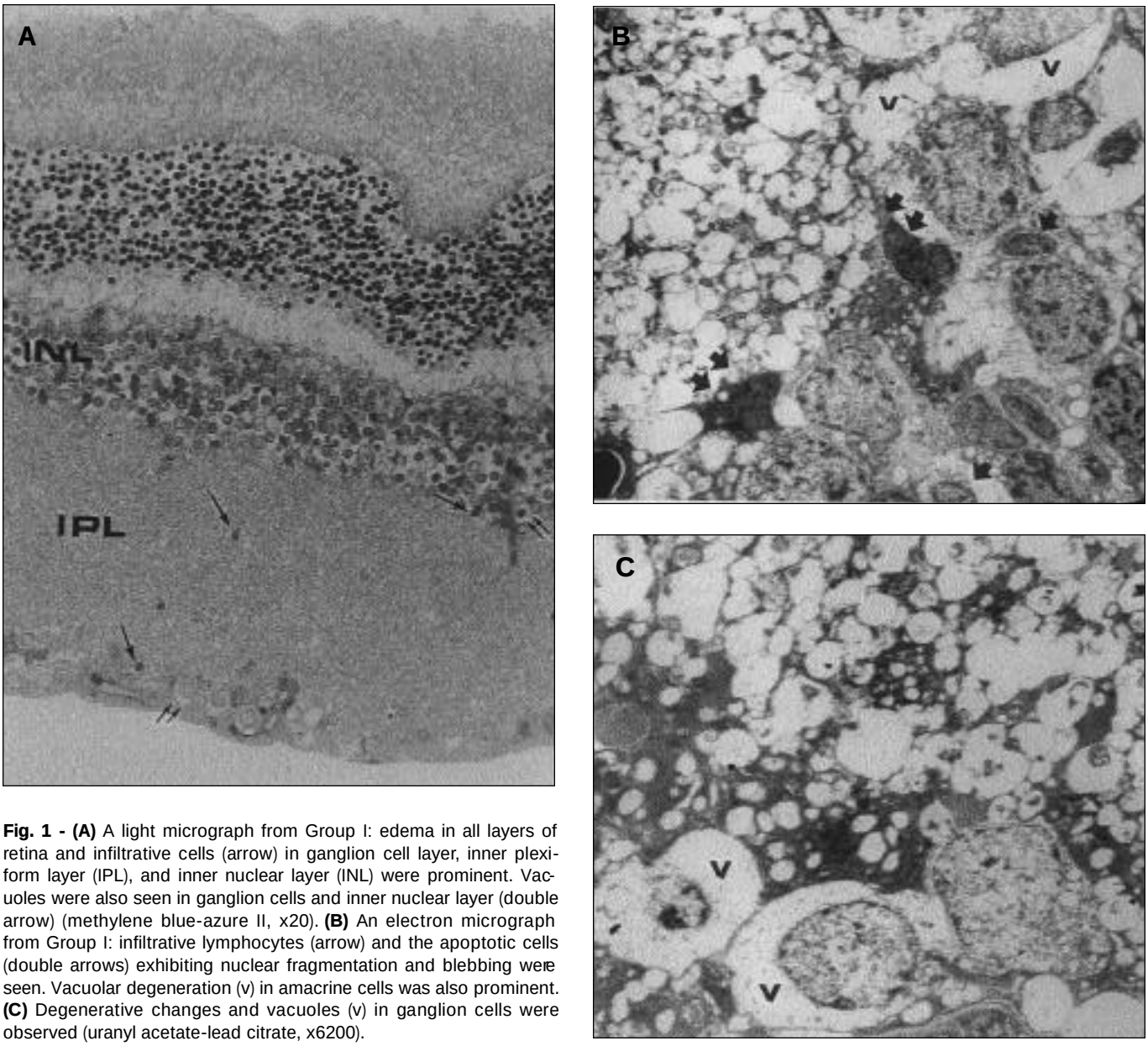


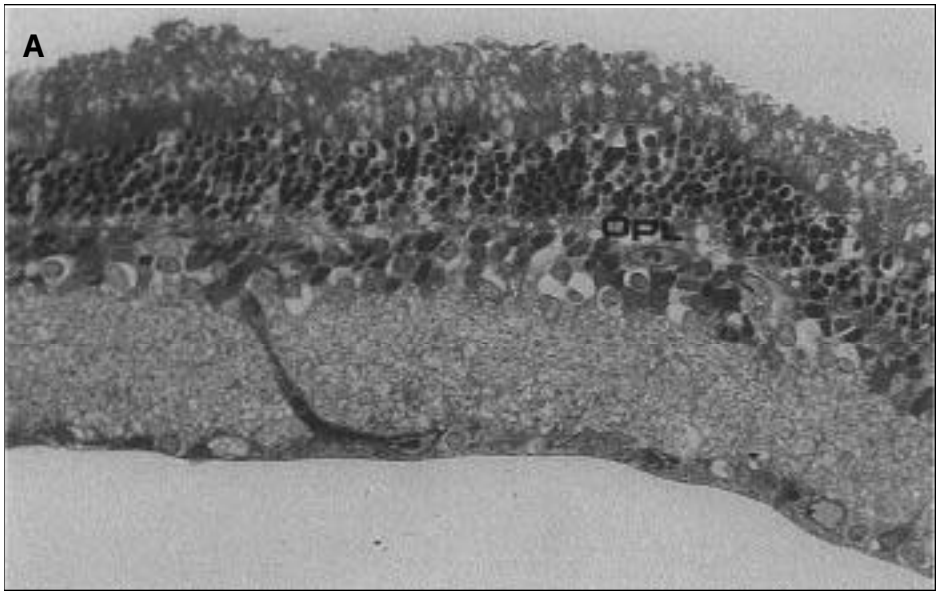
Fig. 1 - (A) A light micrograph from Group I: edema in all layers of retina and infiltrative cells (arrow) in ganglion cell layer, inner plexiform layer (IPL), and inner nuclear layer (INL) were prominent. Vacuoles were also seen in ganglion cells and inner nuclear layer (double arrow) (methylene blue-azure II, x20). **(B)** An electron micrograph from Group I: infiltrative lymphocytes (arrow) and the apoptotic cells (double arrows) exhibiting nuclear fragmentation and blebbing were seen. Vacuolar degeneration (v) in amacrine cells was also prominent. **(C)** Degenerative changes and vacuoles (v) in ganglion cells were observed (uranyl acetate-lead citrate, x6200).

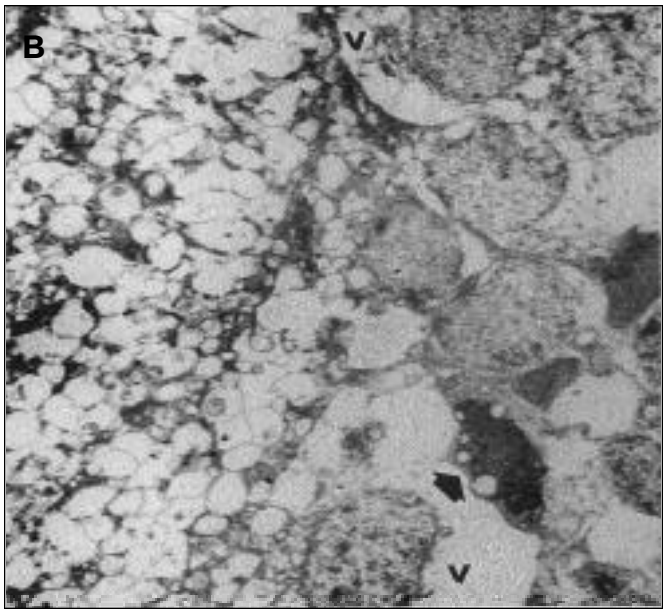
tissue injury becomes worse after perfusion is called reperfusion injury (1). Infiltrating leukocytes are thought to play a major role in ischemia/reperfusion (I/R) injury (1-3). Formation of toxic reactive oxygen species, lipid peroxidation of cellular membranes, and release of vasoactive agents like complement components and prostaglandins have also been reported to be important factors in the pathophysiology of this condition (1, 2).

Thrombin is the terminal serine protease of the coagulation pathway, and has recently been implicated in the inflammatory cascade, particularly to enhance neutrophil recruitment (4). Antithrombin III (AT III), on the other hand, is an I_2 globulin and is the physiologic inhibitor of thrombin and other serine proteases. It has recently been reported that AT III provides protection against I/R injury by inhibiting leukocyte recruitment through suppression of leukocyte-endothelial interaction in postischemic tissues (5).

The aim of the present study was to investigate the effect of AT III on retinal I/R injury in rats.

The retinal I/R injury was obtained by four-vessel





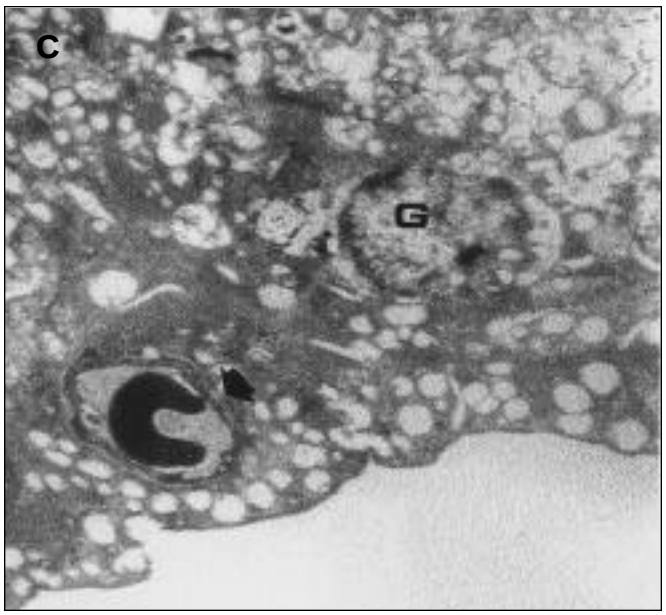


Fig. 2 - (A) A light micrograph from Group II: less edema compared to Group I was observed. The thickness of outer plexiform layer (OPL) was almost normal (methylene blue-azure II, x20). **(B)** An ultrastructural appearance of inner nuclear layer from Group II. Most of the cells and their intercellular junctions were intact. However, vacuolar (V) changes were observed in ama-crine cells. An apoptotic cell with blebs was seen (arrow). **(C)** An almost normally appearing ganglion cell (G) with its nucleus and prominent nucleolus was seen close to the blood vessel (arrow) (uranyl acetate-lead citrate, x6200).

occlusion method in which vertebral and carotid arteries were occluded bilaterally.

MATERIALS AND METHODS

Animals

This study was performed on 10 Wistar albino rats (20 eyes), weighing 250 to 350 g, obtained from Hifzisihha Institute Animal Laboratory, Ankara, Turkey. The rats were housed in wire-bottomed cages at room temperature with 12-hour light-dark cycle. All animals were fed with standard rat chow. The care and handling of the animals were in accordance with the National Institutes of Health guidelines.

Surgical method

Four-vessel occlusion method, which was introduced by Pulsinelli and Brierley (6) and Sano et al (7) to induce

retinal ischemia, was used in this study. After an overnight fast, rats were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar, Parke-Davis, Eczacibasi, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer, Germany). Following skin shaving and preparation with 10% povidone-iodine solution, a 2-cm incision was made behind the occipital bone directly overlying the first two cervical vertebrae. The vertebral arteries were occluded bilaterally by coagulating through alar foramina of the first cervical vertebra with an electric needle coagulator under an operating microscope. The incision was closed with interrupted 4-0 silk sutures.

Forty-eight hours after the initial procedure, the rats were re-anesthetized and a 3-cm midline cervical incision was performed. Both common carotid arteries were exposed and a nontraumatic microvascular clamp was placed around each artery to interrupt blood flow. The absence of blood flow in the retina was monitored by direct ophthalmoscope. Carotid artery clipping time (ie, duration of ischemia) was 30 minutes. At the end of this period, microvascular clamp was removed for the reperfusion of the eye. The reperfusion time was 4 hours. Retinal reperfusion was confirmed by direct ophthalmoscopy of the retinal vessels. The cervical incision was closed with interrupted 4-0 silk sutures. At the end of reperfusion period, the animals were killed by decapitation. The eyeballs were enucleated and were fixed in 2.5% glutaraldehyde solution for retinal preparation.

Experimental Group designs

Rats were divided into two groups, each containing five animals (10 eyes): Group I (control group) and Group II (AT III group). Both groups underwent the same surgical procedure outlined above. In Group II, rats were injected intravenously with 250 U/kg of AT III (Kybernin P human antithrombin III, Farmatek, Istanbul, Turkey) 5 minutes before the induction of ischemia.

Histopathologic evaluation

Tissue samples were fixed by immersion in 2.5% glutaraldehyde in phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer. After dehydrating in ethanol in ethanol gradients at room temperature, the tissue samples were embedded in araldite. Semithin (1 µm thick) and thin (70 nm thick) sections were cut, examined, and photographed under light and electron microscopes (Zeiss EM9-S2). Semithin sections were stained with methylene blue-azure II, and ultrathin sections were stained with uranyl acetate and lead citrate.

The signs of I/R injury at the microscopic level, i.e., cellular degeneration, vacuolization between retinal layers, increase in the retinal thickness due to edema, mononuclear cell infiltration, and apoptotic cells, were recorded for each group. Thickness measurements of the retinal layers (inner plexiform, inner nuclear, and ganglion cell layers) were performed by means of a compass on the photographs taken under light microscope. Apoptotic cell and mononuclear cell counts were performed according to their mean value of three different regions under x40 magnification.

Statistical analysis was performed using Mann-Whitney U test and a p value less than 0.05 was accepted as statistically significant.

RESULTS

Group I

In tissue samples obtained from the rats in the control group, edema in all layers of retina was prominent. Vacuolization due to edema was observed in the outer nuclear layer (ONL) and between the inner and outer segments of photoreceptors under light microscope. Prominent erythrocyte infiltration was also noted between the nuclei of ONL and inner nuclear layer (INL). Increase in the thickness of outer plexiform layer (OPL) and inner plexiform layer (IPL) due to edema and vacuolization in some ganglion cells within the ganglion cell layer (GCL) were also observed. Mononuclear cell infiltration, which was prominent in IPL and GCL, was noted (Fig, 1A).

Mean (± SD) thickness values of the INL, IPL, and GCL were 104.0 ± 2.54 µm, 134.8 ± 10.13 µm, and 151.4 ± 20.7 µm, respectively (Tab. I).

Mean (± SD) values for mononuclear cell infiltration and apoptotic cell counts are given in Table II.

Electron microscopy revealed edema between the axons of IPL. Vacuolizations within the cells of INL, disruption between the intercellular adhesions, and mononuclear cells were observed in the INL. Presence of apoptotic cells was also remarkable (Fig. 1B). Vacuolar appearance around the nuclei and degeneration in ganglion cells and edema in axons were also prominent (Fig. 1C).

Group II

Semithin sections obtained from the retinas of the rats that received AT III revealed a well preserved retinal structure under light microscope (Fig. 2A). GCL layer was observed to be almost normal. INL and IPL layers were similarly well protected; however, some regions showed minimal thickness increment indicating tissue edema. Cells in the ONL were relatively well preserved, and erythrocytes in ONL and INL were not observed in contrast to Group I. Mean (± SD) thickness values of the INL, IPL, and GCL were 39.0 ± 15.67 µm, 90.50 ± 25.39 µm, and 115.0 ± 27.89 µm, respectively (Tab. I).

Mean (± SD) values for mononuclear cell infiltration and apoptotic cell counts are given in Table II.

Electron microscopic evaluation revealed that the axons of the IPL were almost normal in caliber. Some of the cells within INL demonstrated cytoplasmic vacuolizations, and some demonstrated apoptotic changes including nuclear fragmentation and blebbing; however, most of the intercellular adhesions were intact at this layer (Fig. 2B). The degenerative changes in ganglion cells were not observed in most of the cells. The ganglion cells with their prominent nucleolus and pale nucleus have the appearance of a normal ganglion cells (Fig. 2C).

Statistical evaluation

The mean thickness values of the two groups were compared with each other; the difference was found to be significant with respect to INL and IPL (p=0.009 and p=0.012, respectively). Although the average GCL thickness measurement in the control group was higher compared to AT III group, the difference did not reach statistical significance (p=0.074). These results indicate increased retinal thickness measurements in Group I due to tissue edema resulting from I/R injury. Similarly, mononuclear cell infiltration and apoptotic cell counts were observed to be significantly higher in the control group compared with AT III group (p=0.015 and p=0.004, respectively), indicating inhibitory effect of AT III on leukocyte infiltration and apoptotic cell death in rat retina.

DISCUSSION

The present study demonstrates the protective effects of AT III supplementation given 5 minutes before ischemia induction on retinal ischemia/reperfusion injury in rats in terms of histopathologic changes.

Thrombin, other than its function in coagulation cascade, was shown to have a role in neutrophil—endothelial interaction, which is the initial step for leukocyte recruitment in inflammation. Thrombin and its 14-amino acid peptide fragment (thrombin receptor activated peptide, TRAP-14) increase the expression of endothelial P- and E- selectin (4, 8). Additionally, thrombin induces expression of intercellular adhesion molecule-1 (ICAM-1) (9). Selectins and ICAM-1 are critical molecules in the process of leukocyte infiltration into the inflamed tissues

TABLE I - MEAN ± SD THICKNESS VALUES OF RETINAL LAYERS IN BOTH GROUPS

	INL	IPL	GCL
Group I	104.0 ± 2.54 µm	134.8 ± 10.13 µm	151.4 ± 20.7 µm
Group II	39.0 ± 15.67 µm	90.50 ± 25.39 µm	115.0 ± 27.89 µm
p	0.009	0.012	0.074

INL = Inner nuclear layer; IPL = Inner plexiform layer; GCL = Ganglion cell layer

TABLE II - MEAN ± SD VALUES FOR MONONUCLEAR CELLULAR INFILTRATION AND APOPTOTIC CELL COUNTS UNDER LIGHT MICROSCOPE (x40) IN BOTH GROUPS

	Cellular infiltration score	Apoptotic cell score
Group I	6.67 ± 1.97	18.2 ± 5.7
Group II	3.66 ± 1.03	8.66 ± 2.3
p	0.015	0.004

(4). In the first step of leukocyte infiltration, leukocytes tether and roll along the vascular endothelial cells, and endothelial P-selectin is essential for this step. Then, some of the rolling leukocytes adhere to the endothelium via I_2 integrins and ICAM-1. In the final stage, leukocytes leave the circulation and enter the tissues (emigration). Infiltrating leukocytes have been implicated as the key mediators of I/R injury (1). These activated leukocytes release toxic oxygen products, proteases, and elastases, resulting in tissue injury.

AT III is an I_2 globulin and inhibits serine proteases such as thrombin, factors Xa, IXa, XIIa, and kallikrein. It was reported that AT III protects against I/R injury in an in vivo feline intestinal model through the suppression of leukocyte infiltration into the postischemic tissues (5). Other than intestines, protective effects of AT III on I/R injury have been described in the liver, lung, and kidney of various animal models (10-12). A recent study investigated the inhibitory effects of AT III on interactions between blood cells and endothelial cells during retinal I/R injury in rats (13). AT III administration significantly inhibited leukocyte rolling along retinal veins and subsequent accumulation of leukocytes in the postischemic retina. Additionally, it was immunohistochemically shown that P-selectin and ICAM-1 expression was suppressed by AT III. Histologic examination of the rat retinas revealed that the AT III-treated rats had less damaged retinal tissues. It was suggested that AT III reduces I/R injury by suppressing the expression of adhesion molecules (P-selectin, ICAM-1), which results in inhibition of leukocyte and platelet accumulation in postischemic retina, and hence reduces retinal damage (13). However, other studies reported that AT III might prevent I/R injury by increasing the levels of prostacyclin (prostaglandin I2) (10). AT III was shown to promote the endothelial release of PGI2 by interacting with cell-surface glycosaminoglycans (14). Prostacyclin is a potent inhibitor of platelet aggregation and leukocyte activation, and has vasodilatory action. Due to its biological actions, prostacyclin may also have a role in the protective effects of AT III on retinal I/R injury.

Our study had similar findings to those of Nishijima et al (13) in terms of histopathologic findings. Leukocytic infiltration into retina was significantly reduced in rats that received AT III. Retinal thickness values, an indicator of edema due to I/R injury, were measured to be considerably low in AT III group. In our study, after 4 hours of reperfusion, animals were killed and retinas were harvested. Since retinal edema is taken as a severity measurement of I/R injury, retinas obtained from control group had more retinal edema and thus, had thicker retinal layer measurements. On the other hand, reperfusion period was 14 days in the study by Nishijima et al (13), and retinas were obtained at the end of this period. So, retinal atrophy replaced the early edema during this time, resulting in thin and atrophic retinas in rats that did not receive AT III. In our study, additionally, apoptotic cell counts were found to be significantly lower in AT III group compared to controls.

In conclusion, AT III attenuated I/R injury in rat retina. The present study demonstrates the protective effects of AT III against I/R injury in rat retina in terms of histopathologic changes, retinal thickness measurements, mononuclear cell infiltration, and apoptotic cell counts. Retinal structure was found to be well preserved in AT III-treated group when compared to controls. AT III may be an effective agent in the prevention or treatment of retinal I/R injury.

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