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Aprotinin reduces ischemia-reperfusion injury in the retina of guinea pigs

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PURPOSE. *The aim of this study was investigate the role of aprotinin on retinal lipid peroxidation and histopathological changes during ischemia/reperfusion (I/R) of guinea pigs.*
METHODS. *Three groups of seven pigmented guinea pigs each were formed: a control (group 1), ischemia/saline (group 2) and ischemia/aprotinin (group 3). One eye of each animal was selected for histopathological evaluation and the other for biochemical assay. Bilateral pressure-induced retinal ischemia was instigated for 90 min and was followed by 24 hours of reperfusion. Animals in the ischemia/aprotinin and ischemia/saline groups received either 20,000 KIU/kg of aprotinin or saline, repeated four times at 6-hour intervals, with the first dose administered 5 min prior to the ischemic insult. The animals were killed at 24 hours of reperfusion. Retinal malondialdehyde (MDA) levels and the thickness of the inner plexiform layers were measured.*
RESULTS. *The level of MDA in group 1 was significantly ($p<0.001$) lower than the other groups. The mean MDA level in group 2 was significantly ($p<0.01$) higher than in group 3. The inner plexiform layer in group 1 was significantly ($p<0.001$) thinner than in the other groups. The mean thickness of the inner plexiform layer in group 2 was significantly ($p<0.01$) higher than in group 3.*
CONCLUSIONS. *These data indicate that intraperitoneally administrated aprotinin has a protective effect against I/R injury in the retina of guinea pig as evidenced by reduced retinal MDA level and retinal thickness. (Eur J Ophthalmol 2003; 13: 642-7)*

KEY WORDS. *Ischemia-reperfusion injury, Retina, Aprotinin*

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INTRODUCTION

Ischemia/reperfusion (I/R) injury is no stranger to the human retina (1). Retinal ischemia is a final common pathway for injury in many diseases that result in blindness. The condition is encountered in retinal artery and vein occlusions, diabetes, sickle-cell disease, inflammatory diseases, and perhaps most dramatically in retinopathy of prematurity, where the tragic consequences of both ischemia and reperfusion injury are apt to occur (2). Therefore, a number of model systems have been developed for examining the structural and functional defects associated with ischemia. The intraocular pressure-induced ischemia in mammals is the most frequently used model to investigate mechanisms and possible therapy for retinal ischemia (3, 4). The degree of insult to the retina is dependent on the animal species, duration

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and magnitude of the increase in intraocular pressure (IOP) (5). Restoration of blood flow to ischemic retina can result in recovery of cells if they are reversibly injured, or not affect the outcome if reversible cell damage has occured (6). However, depending on the intensity and duration of the ischemic insult, a variable number of cells may proceed to die after blood flow resumes, by necrosis, as well as by apoptosis (7, 8).

Aprotinin is a broad-based serine proteinase inhibitor isolated from bovine lung. It is a serine protease inhibitor that is presently widely used for minimizing perioperative blood loss in cardiac operations (9). Because proteolytic enzymes play an important role in ischemic retinal damage, one may expect a protective effect of aprotinin on the ischemic retina. Experimental studies have shown that aprotinin, beyond its antiproteolytic membrane stabilizing property, decreases the release of lysosomal enzymes and increases intracellular adenine nucleotides (10, 11). While aprotinin reduces reperfusion injury by supressing bradykinin, it can also inhibit the production of superoxides and peroxides which originate from human leukocytes (10-12).

The mechanisms involved in ischemic retinal degeneration are still poorly understood, and there are no current efficacious therapies. We hypothesized that aprotinin can provide protection against the adverse effects of reperfusion in the retina. The aim of the present study was to investigate the effect of aprotinin on I/R-induced retinal injury in guinea pigs.

METHODS

Animals

All experiments adhered to the ARVO statement for the use of animals in ophthalmic and vision research. Twenty one pigmented male guinea pigs 5 to 6 months of age, each weighing 500-600 g, were used in the experiments. The animals were classified randomly into 3 groups in equal numbers (n=7): group 1 as control, group 2 ischemia/saline group and group 3 ischemia/aprotinin. The animals were anesthetized by intramuscular injection of 50 mg /kg ketamine hydrochloride (Ketalar®, Eczacıbaşı, Turkey) and 5 mg/kg xylazine (Rompun®, Bayer, Turkey). They were housed in a room with a 12-hour light/dark cycle and allowed free access to food and water.

Animals in the ischemia/aprotinin and ischemia/ saline groups received intraperitoneal injections of either 20,000 KIU[1] aprotinin/kg (Trasylol®, Bayer, Germany) or saline, repeated four times at 6-hour intervals, with the first dose administered 5 min prior to the ischemic insult. I/R was not induced nor was any medication used in the animals of group 1.

Induction of ischemia and reperfusion

The anterior chambers of each animal were cannulated with a 27-gauge infusion needle connected to a bottle containing normal saline. Retinal ischemia was induced by raising the saline reservoir to a height of 204 cm, thereby increasing the IOP to 150 mmHg. The high IOP was maintained for 90 minutes. At this IOP, systolic pressure collapse of the central retinal artery was observed by direct ophthalmoscopy. After the ischemia, reperfusion was performed for 24 hours. At the end of the reperfusion period, the animals were killed with an intracardiac injection of 50 mg/kg sodium pentobarbital (Pentothal Sodium®, Abbott). The eyes were enucleated quickly under the microscope. One eye of each animal was selected for biochemical assay and the other for histopathologic analysis.

Preparation of tissue samples for biochemical assay

The eyeball was cut posteriorly, to the limbus and after excision of the anterior segment, the vitreous was removed, then the retina was carefully dissected from the sclera. The wet weight of the retina was measured within one minute. Then the retina was incubated in cold TE buffer (10 mM Tris-HCl, 0.67 mM acetylsalicylic acid, 0.5 mM EGTA and 0.1 mM butylated hydoxytoluene, pH 7.4). The isolated retina was placed on dry ice immediately and kept frozen at –80 °C till assay.

[1] Kallikrein inactivator units. 100,000 KIU is 14 mg protein. Fifty-milligram ampules of Trasylol contain 10,000 KIU/ml = 500,000 KIU (70 mg)

Malondialdehyde (MDA)

Retinal samples were conicated (Vibra Cell, Danbury, USA) with three 20-second bursts in an ice bath. MDA in retinal homogenate was measured spectrophotometrically at 535 nm as the product of the reaction with thiobarbituric acid using a modification of the method of Dahle et al (13). Standards were prepared from different dilutions of 1,1', 3,3'-tetraethoxypropane (Sigma Chem Co, USA). Results were expressed in nmol/100 mg tissue wet weight.

Preparation of tissue samples for histopathologic analysis

The eyes were cut open and fixed in 10% formaline. The samples were then dehydrated, embedded in paraffin, sectioned with a microtome at 4-µm thickness, and stained with hematoxylin and eosin. Each section was cut along the horizontal meridian of the eye through the optic nerve head. Sections were cut perpendicular to the retinal surface. Retinal sections were examined with an optical microscope (Olympus BX50, Tokyo, Japan) at 200 x magnification.

Histological changes

It has been demonstrated previously that changes in retinal layer thickness accurately reflect changes in cell number (14). I/R-induced cell swelling is well-recognized and documented in the inner plexiform layer of the retina (15,16). To quantify the retinal damage induced by I/R injury, we measured changes in thickness of inner plexiform layer, according to the method described by Hughes (4), with slight modification. The thickness of this layer in each section was measured in the retina at a distance of 0.5 cm from the center of the optic nerve head. Three measurements were obtained from adjacent locations in each nasal and temporal hemisphere. The value of each retinal thickness was averaged from six measurements of five sections from each eye (Fig.1).

Statistical analysis

Statistical calculations were done by using SPSS (version 6.1; SPSS Inc. Chicago). The results were expressed as the mean ± SD. Differences between

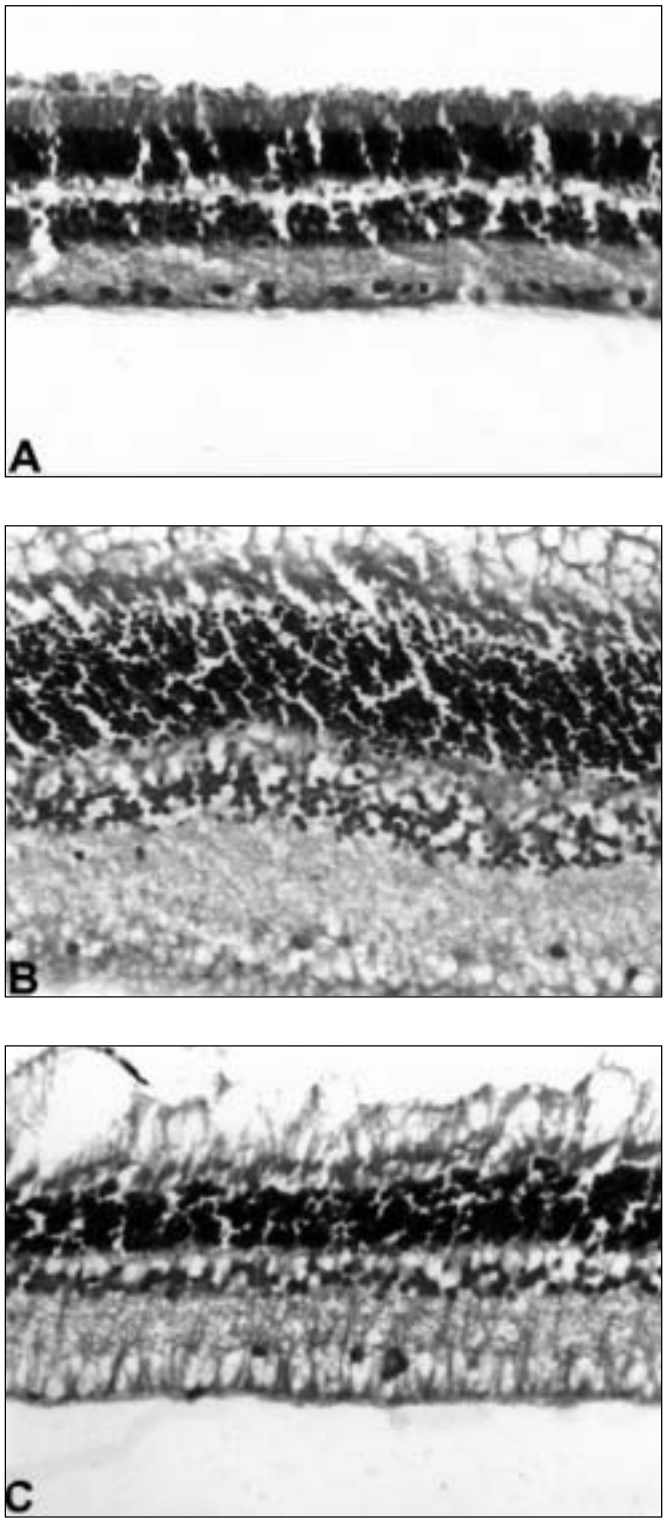

Fig. 1 - *Paraffin sections of the guinea pig retina. The mean thickness (edema formation) of the inner plexiform layer for each eye was measured.* **A)** *Control;* **B)** *Ischemia/saline administrated group;* **C)** *Ischemia/aprotinin administrated group Hematoxylin and eosin x 200.*

groups were evaluated using the Kruskal-Wallis test and differences were analysed by Tukey's B-test. A p value <0.05 was considered statistically significant.

RESULTS

The mean MDA contents are set out in the Table I. The content of MDA in the normal retina was significantly (p<0.001) lower than in the other groups. The mean MDA level was 5.9 times higher in group 2 compared with group 1 (p<0.001). The mean MDA level was 24.3 ± 4.8 nmol/100 mg tissue wet weight in the group 2. This was significantly (p<0.01) higher than in the group 3.

The mean thickness of the inner plexiform layers are set out in the Table II. The inner plexiform layer in group 1 (control) was significantly (p<0.001) thinner than in the other groups. The mean thickness of the inner plexiform layer was 23.9 ± 1.8 μm in the group 2. This was significantly (p<0.01) higher than in the group 3.

Histologic sections demonstrated that tissue damage in ischemic guinea pigs was most obvious in the inner retina. In particular, retinal edema was well-recognized in the inner plexiform layer (Fig.1). The retinal tissues of the guinea pigs that had been treated with saline exhibited significant changes of the inner retinal layers. This was characterised by edema of the inner plexiform layer and degenerative changes of the ganglion cell layer. By contrast, in the aprotinin-treated guinea pigs, the retinas had mild changes.

DISCUSSION

Despite a great deal of research on the subject of retina protection and the prevention of I/R injury in retina, consensus has not been reached on suitable pharmacological agents. In this study, in the light of physiopathology of damage due to reperfusion injury, we used aprotinin in an ischemic animal model. The results of this study indicate that MDA levels in the retina were higher in the ischemia/saline group than in the control group and lower in aprotinin administrated group than in the ischemia/saline group. In addition, retinal thickness in the ischemia/saline group was higher in the control group and lower in the aprotinin administrated group.

Oxygen free radical-induced oxidation can cause severe cell damage in biological systems. These radicals have been implicated in many ocular diseases, such as uveitis, light damage, and I/R injury (17-19). The outer segments of retinal photoreceptors have a high content of long-chain polyunsaturated fatty acids, which render the retina very susceptible to oxidative damage (18, 20). Oxidative damage to retinal cell membranes adversely affects their normal physiology and may lead to sight-threatening ocular diseases (21). Living organisms have developed a wide array of antioxidant defences to prevent free radical formation or limit the damaging affects. Experimental studies have shown that lipid peroxidation is an event caused by imbalance between free radical production (2, 19). Higher levels of MDA, a final product of the lipid peroxidation process, have been observed in I/R injury. High retinal levels of MDA are probably due to continuous stress dependent discharge of

TABLE I - MALONDIALDEHYDE (MDA) LEVELS IN RETINAL TISSUE OF GUINEA PIGS

Control n=7	Ischemia/saline n=7	Ischemia/aprotinin n=7
4.1 ± 1.7	24.3 ± 4.8 [a]	12.4 ± 1.9 [a, b]

MDA levels are expressed as nmol/100 mg tissue weight and mean ± SD
[a] vs control at p<0.001
[b] vs ischemia/saline group at p<0.01

TABLE II - THE MEAN THICKNESS OF THE INNER PLEXIFORM LAYER IN GROUPS

Control n=7	Ischemia/saline n=7	Ischemia/aprotinin n=7
12.7 ± 0.7	23.9 ± 1.8 [a]	18.4 ± 0.8 [a, b]

Values are expressed μm and mean ± SD
[a] vs control at p<0.001
[b] vs ischemia/saline group at p<0.01

ischemia-reperfusion injury. Ischemia is caused by hypoxia. In association with additional insults cause by re-oxygenation (reperfusion), the ischemia-reperfusion may lead to eventual retinal tissue degeneration by the hyper production of toxic oxyradicals. Therefore, increased retinal thickness was supported by our increased MDA levels.

Many enzymes in catalytic cycles in an ischemic environment cause the formation of free radicals (2). Of these, xanthine oxidase, which is the most studied enzyme, forms superoxide radicals while transforming from its xanthine dehydrogenase form to an oxidase form in ischemia (22). Reductions in cell energy production and defective membrane functions cause intracellular calcium levels to rise due to ischemia. High intracellular calcium concentrations activate protease, which transforms xanthine dehydrogenase into xanthine oxidase (23). Aprotinin, a protease enzyme inhibitor, inhibits the transformation of xanthine dehydrogenase into xanthine oxidase, thus preventing free oxygen radical formation (24). MDA is a good indicator of free oxygen radical formation. MDA elevation shows increased lipid peroxidation due to the effects of free oxygen radicals (25). The level of MDA in the I/R plus aprotinin group was significantly lower than in the I/R plus saline group. Other researchers (10, 11) have also established that aprotinin reduces MDA levels.

While aprotinin inhibits the free oxygen radical formation, it can also inhibit bradykinin, kallidin and other kinins (9). Bradykinin, kallidin and other kinins (a known inflammatory mediators) have high activity as permeability factors (26). Experimental studies have shown that aprotinin, beyond its antiproteolytic membrane stabilizing property, decreases the release of lysosomal enzymes and increases intracellular adenine nucleotides (27). Histologic sections in the study described herein showed the protective effects of aprotinin in retinal I/R injury. To our knowledge, aprotinin has been used in I/R-induced retinal injury for the first time here. Aprotinin reduced retinal thickness due to its protective effects on inflammatory reactions.

In conclusion, we found that an aprotinin supplement was related to a decreased risk of progression of I/R damage in guinea pig retina. The findings obtained with aprotinin in the present study may be related to decreased lipid peroxidation, leukocyte activation, protease release and agents like cytokines inducing inflammatory response. However, we believe that the effect of aprotinin on retinal tissue during I/R needs to be further evaluated. We used recommended doses in our study (27). Therefore, further experiments with lower and/or higher doses of aprotinin need to be performed to obtain more reliable results.

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