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Protective effects of intraperitoneal vitamin C, aprotinin and melatonin administration on retinal edema during experimental uveitis in the guinea pig

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A considerable amount of clinical and experimental evidence exists suggesting the involvement of reactive oxygen substances (ROS) in the aetiology of uveitis. The activated phagocytic system of polymorphonuclear leucocytes in uveitis is involved in the generation of ROS. In addition to their direct free radical scavenging action, aprotinin, melatonin and vitamin C are known to protect against oedema formation and can preserve plasma membrane fluidity and free radical production. Histological changes in the retina that occur during uveitis are not well explained. The purpose of this study was to determine whether vitamin C, aprotinin and melatonin can protect the retina from damage accompanying experimental uveitis (EU). Thirty adult male guinea pigs were divided into five groups of six animals each. The first group was used as control. The right eyes of groups 2, 3, 4 and 5 received an intravitreal injection of bovine serum albumin for induction of experimental uveitis. At the same time and also on the consecutive third day, groups 3, 4 and 5 received intraperitoneal injections of vitamin C (ascorbic acid, 100 mg kg^{-1} body wt), aprotinin (20 000 kIU kg^{-1} body wt) and melatonin (10 mg kg^{-1} body wt), respectively. The animals were killed on the sixth day. The average thickness of the retina and inner plexiform layer for each eye was measured in sagittal section near the optic nerve and expressed in microns. The thickness of the retina and inner plexiform layer in the control group was significantly ($p < 0.01$) lower than in the group EU as compared with the group EU plus vitamin C, group EU plus aprotinin, group EU plus melatonin ($p < 0.05$). The thicknesses of the retina and inner plexiform layer in group EU plus vitamin C, group EU plus aprotinin and group EU plus melatonin were significantly ($p < 0.01$) lower than that in the group EU. The difference in thickness of the retina and inner plexiform layer among the groups 3, 4 and 5 was not significant ($p > 0.05$). In conclusion, this study demonstrated that oedematous effects of EU on the retina were reduced by the administration of intraperitoneal vitamin C, aprotinin and melatonin, i.e. these antioxidants had significant protective effects on the retina of guinea pigs against oedematous damage in EU. However, the reductive effect of vitamin C on EU was greater than that of aprotinin and melatonin. The intraperitoneal vitamin C, aprotinin and melatonin supplementations may strengthen the antioxidant defence system because of decreased ROS, and these agents may play a role in treating uveitis.

KEY WORDS — experimental uveitis; retina; vitamin C; aprotinin; melatonin

ABBREVIATIONS — EU, experimental uveitis; BSA, bovine serum albumin; ROS, reactive oxygen substances; PMN, polymorphonuclear leucocytes

INTRODUCTION

Uveitis is a complex, acute or chronic inflammatory process that primarily involves the uvea. The processes can extend, however, to involve the retina,

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intraoculer cavities, optic nerve and other optic structures.[1] Chronic intraoculer inflammation is a major cause of blindness. This loss of vision is the result of damage inflicted by inflammatory cell infiltration, particularly the release of various mediators including oxygen metabolites.[1,2] In uveitis, the primary inflammatory cellular infiltration consists of an admixture of phagocytes and various types of lymphocytes.[3] The former includes polymorphonuclear leucocytes (PMN) and macrophages, which on activation are known to generate reactive oxygen substances (ROS).[4–6]

ROS can cause severe membrane injury by initiating various reactions, including lipid peroxidations, which may alter membrane integrity and permeability characteristics.[1] The outer segments of retinal photoreceptors have a high content of long-chain unsaturated fatty acids, which render the retina very susceptible to oxidative damage.[7] Oxidative damage to retinal cells adversely affects their normal physiology and may lead to sight-threatening ocular diseases.[5] Oxidative damage also causes tissue degeneration in the eye if it is not well provided with eye antioxidants.[8] In experimental uveitis, analysis of retina revealed the presence of lipid peroxidation products.[6–8]

The balance between the production and catabolism of oxidants by cells and tissue is critical for maintaining the biological and structural integrity of retina and other intraocular structures.[8] In the physiological state, antioxidants and free radical scavengers may protect the eye by trapping radicals or by interfering with oxidative chain reactions.[1] However, it is possible that these protective agents may be overwhelmed when abundant ROS are generated during uveitis.[5] The overwhelming presence of the ROS may lead to structural damage in the retina, optic nerve, lens and anterior segment.[1,6,8] Studies suggest that in pathological conditions such as uveitis, native antioxidant levels offer optimal protection against free radicals. Moreover, the intracellular distribution of various antioxidant enzymes may not protect the extracellular structures of eye from free radicals liberated at extracellular sites; water-soluble and lipid-soluble antioxidants may play a role in suppressing the damaging effects of the oxidants.[8,9] However, whether vitamin C, melatonin and aprotinin affect oedema in guinea pigs with uveitis is currently unknown and warrants further study.

Vitamin C is a well-known cell protective natural antioxidant.[9] The protective effects of vitamin C are observed in oxygen-dependent pathophysiological conditions.[10,11] Aprotinin is a broad-based serine proteinase inhibitor isolated from bovine lung.[12] Experimental studies have shown that aprotinin, in addition to its antiproteolytic membrane-stabilizing property, decreases the release of lysosomal enzymes, increases intracellular adenine nucleotides and has an anti-inflammatory effect.[12] Melatonin is produced in the pineal gland and also in the retina.[13,14] The protective effects of melatonin against the destructive actions of free radical-initiated processes have been demonstrated in experimental situations.[15] These protective effects have been shown in the retina.[4] In the retina, melatonin is located at the outer nuclear layer of photoreceptor cells and it may normally have a physiological protective role against retinal lipid peroxidation.[13] Melatonin has been shown to markedly protect both membrane lipids and retinal nuclear DNA from oxidative damage.[14] Melatonin's ability to protect against lipid peroxidation in liver, brain and kidney is histologically obvious in *in vivo* studies.[13,14] Decreased plasma melatonin levels were found in patients with uveitis.[16] Therefore, its administration may protect the retina against damage accompanying to uveal inflammation.

The increased levels of ROS could be due to increased generation or decreased neutralization of these species which may cause certain pathological changes. There is evidence which shows that tissue degeneration in the uveitic eye may be due to free radicals.[5,6] To our knowledge, there is scarce information on the treatment effects of intraperitoneal administration of melatonin, vitamin C and aprotinin on experimental uveitis (EU) although there are reports on antioxidant and oxidant effects of melatonin, vitamin C and aprotinin in the retina. In addition, the identification of naturally-occurring antioxidants and their possible role in chemo-protection provides a strategy that is of great interest for medicine therapy. The present study was undertaken to investigate the possible protective effects of vitamin C, aprotinin and melatonin against EU-induced retinal oedema injury by retinal histological evaluation.

MATERIALS AND METHODS

Animal handling

All studies were carried out in compliance with the statement for the use of Animals in Ophthalmic and Vision Research. Male guinea pigs 5 to 6 months of age, each weighing 500–600 g, bred in our laboratory, were used. Only one eye of 30 guinea pigs was used. Housing was at 22–24°C with light from 08.00 to 20.00 hours with free access to water. Animals were

housed individually in stainless steel cages in a pathogen-free University Laboratory Animal Research facility. All animals were fed a commercial diet during the experiment and allowed free access to food and water. The animals were classified randomly into five groups of six animals each. Group 1 was the control; group 2 was the EU group; group 3, vitamin C (EU plus vitamin C); group 4, aprotinin group (EU plus aprotinin) and group 5, melatonin group (EU plus melatonin). The animals were anesthetized by intramuscular injection of $50\,mg\,kg^{-1}$ BW ketamine hydrochloride (Ketalar®, Eczacibaşi, Turkey) and $5\,mg\,kg^{-1}$ BW xylazine (Rompun®, Bayer, Turkey). The conjunctiva was sterilized with 5% povidone–iodine and irrigated with saline. The pupils were dilated with 2.5% phenylephrine hydrochloride.

Induction of experimental uveitis

The upper temporal sclera of the right eye of each guinea pig was injected using a 27-gauge needle, 2 mm to the rear of the limbus with 100 μg BSA dissolved in 100 μl of 0.9% physiological saline given intravitreally.

Intravitreal injections

EU was induced by BSA administration intravitreally, although compounds were intraperitoneally administrated. The right eyes of groups 2, 3, 4 and 5 received intravitreal injection of bovine serum albumin (BSA) 100 μg $(100\,\mu l)^{-1}$, 0.9% physiological saline for induction of EU. At the same time and on the third day, groups 3, 4 and 5 received intraperitoneally, injections of vitamin C (ascorbic acid, $100\,mg\,kg^{-1}$ BW), aprotinin (20 000 $kIU\,kg^{-1}$ BW) or melatonin ($10\,mg\,kg^{-1}$ BW), respectively. Vitamin C was obtained from Redoxon, Roche, Istanbul, Turkey, aprotinin from Trasylol, Bayer, Heidelberg, Germany, and melatonin from Sigma, MO, USA. The melatonin was dissolved in pure ethanol and kept +4°C. Before infection it was further diluted with physiologic saline.

Preparation of tissue samples

On the sixth day after the injections, the animals were killed with an intracardiac injection of $50\,mg\,kg^{-1}$ BW sodium pentobarbital (Pentothal Sodium®, Abbot, Istanbul). The eyes were enucleated quickly under the microscope. Immediately after the enucleation, the eyes were cut open and fixed in 10% formaline. The eyes were then dehydrated, embedded in paraffin, sectioned with a microtome at 4-μm thickness, and stained with haematoxylin and eosin.

Histological measurement

The average thickness (oedema formation) of the retina and inner plexiform layer for each eye was measured with an ocular micrometer (Olympus BX50, Tokyo, Japan), at 40×10 magnifications in sagittal sections near the optic disc. The measurements were expressed in microns. Six measurements were obtained and averaged for each eye. The microphotographs were taken at 20×10 magnifications for better resolution (Figure 2 a–e).

Statistical analysis

Quantitative data are expressed as mean ± standard deviation (SD). Between groups comparisons were performed with Kruskal–Wallis's test for unpaired comparisons followed by Mann–Whitney's rank sum test. All analyses were performed with SPSS 11.0 for Windows software. P values of <0.05 were considered statistically significant.

RESULTS

The mean thicknesses of the retina are set out in the Figures 1 and 2 a–e. The mean thickness of the retina was 83, 118.5, 90.3, 94.3 and 92.0 μm in the control, EU, EU plus vitamin C, EU plus aprotinin, EU plus melatonin groups, respectively. The thickness of the retina and inner plexiform layer in the control group was significantly ($p < 0.01$) lower than in the other groups, whereas the thickness in the retina and inner

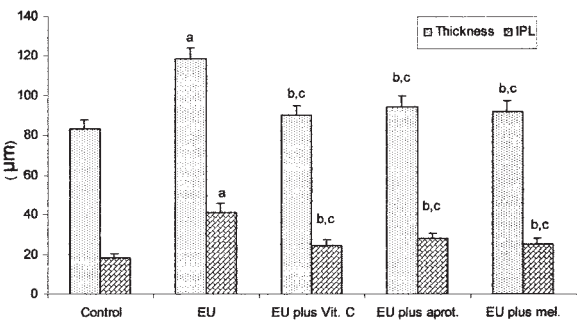

Figure 1. The total thickness and inner plexiform layer (IPL) of the retina in vitamin C, aprotinin and melatonin administered guinea pigs during experimental uveitis (EU). Values in μm are expressed as mean ± SD. a $p < 0.01$, group control versus EU; b $p < 0.05$, group control versus groups: EU plus Vit.C, EU plus aprot. and EU plus mel.; c $p < 0.01$, group EU versus groups: EU plus Vit.C, EU plus aprot. and EU plus mel

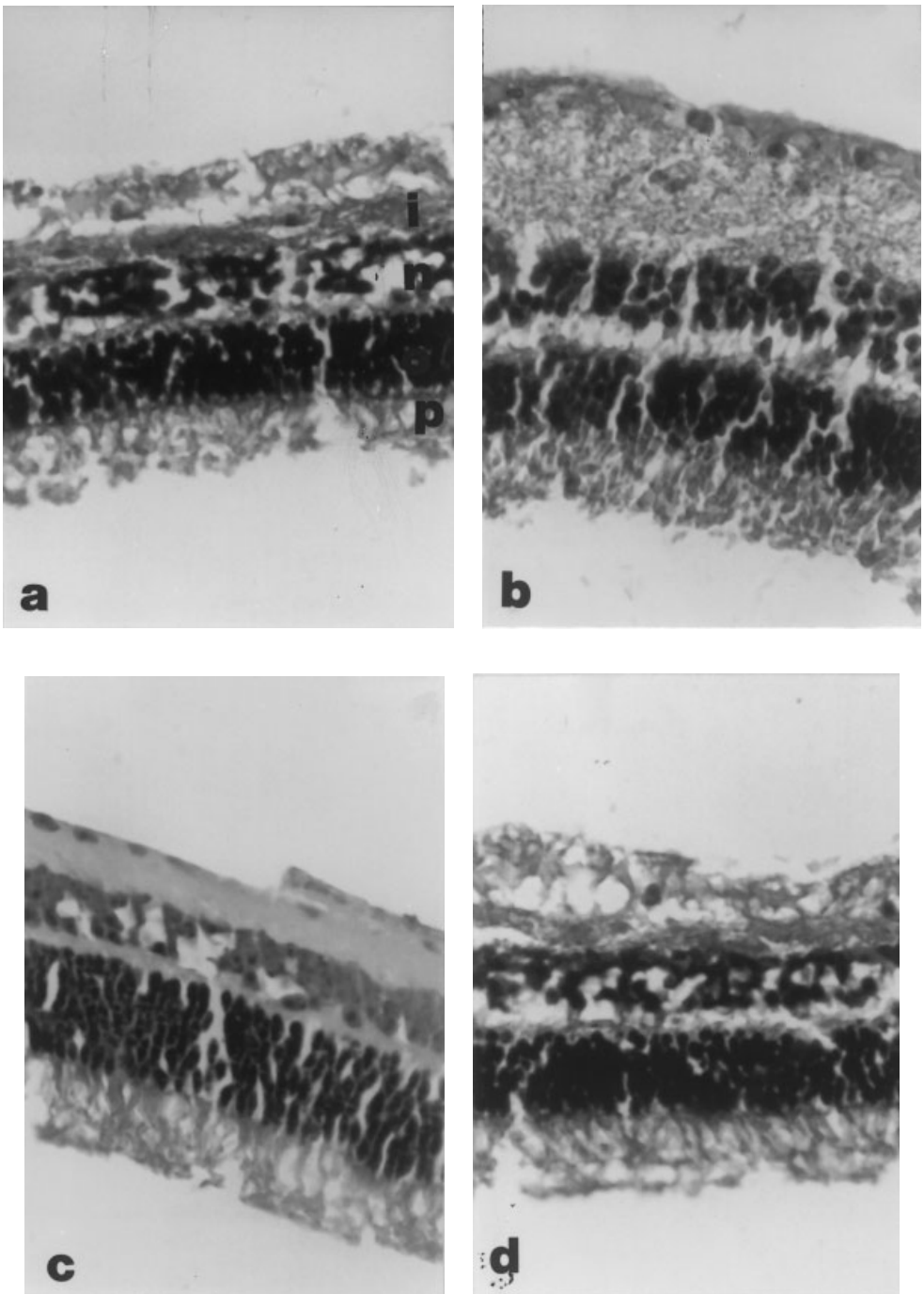

Figure 2. Paraffin sections of the guinea pig retina. The mean thickness (oedema formation) of the retina and inner plexiform layer for each eye was measured in sagittal section near the optic nerve. Haematoxylin and eosin, original magnification 20×10. (a) Control; (b) experimental uveitis (EU); (c) EU + vitamin C; (d) EU + aprotinin; (e) EU + melatonin. **i**, inner plexiform layer; **n**, internal nuclear layer; **o**, external nuclear layer; **p**, photoreceptor layer

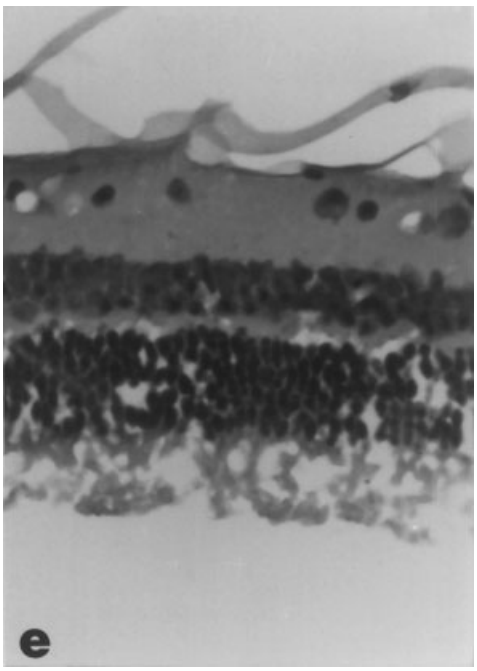

Figure 2. Continued

plexiform layer of groups supplied with vitamin C, aprotinin and melatonin was significantly ($p < 0.01$) lower than that in the EU group. Between the thickness of the retina and inner plexiform layer in the treatment groups (groups 3, 4, 5) there was no significant difference (Figures 1 and 2).

The mean thickness of the inner plexiform layer is shown in Figures 1 and 2. It was 18.3, 41.3, 24.3, 28.0 and 25.1 μm in the control, EU, EU plus vitamin C, EU plus aprotinin, EU plus melatonin groups, respectively. Compared with the control group, histological sections demonstrated that in the EU group of guinea pigs all of the retinal layer was affected but oedema was most obvious in the inner retina. Retinal oedema was particularly well recognized in the inner plexiform layer. Oedema in the inner plexiform layer and degenerative changes of the ganglion cell layer was typical. The retinas of all the three treatment groups had been protected from oedema formation, particularly in the vitamin C and melatonin groups. In the aprotinin group there were some degenerative changes in the ganglion cell layer.

DISCUSSION

Different strategies have been proposed to inhibit uveitis-induced tissue degeneration.[1] Development of therapies to prevent the action or generation of ROS may influence the progression of oxidative eye damage, along with the appearance of uveitis-induced eye damage. Natural antioxidants such as melatonin, vitamin C and aprotinin can be easily and safety increased in the eye by intraperitoneal supplementations. In this study we investigated the effects of intraperitoneal melatonin, vitamin C and aprotinin on retinal oedema during EU in the guinea pig.

Uveitis is a worldwide frequent inflammation of the eye. For experimental investigations EU can be induced using many materials such as BSA, lipopolysaccharide and retinal S-antigen. Induction of EU by a single intraocular injection of BSA is a well-known method.[17,18]

ROS-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 ischemia–reperfusion injury.[1,5,10,11] Elaborate mechanisms exist within cells to reduce the damaging effects of these ROS, but these mechanisms may sometimes be insufficient.[19] In uveitis, free radical-induced oxidative damage plays an important role and antioxidant therapy may be necessary to protect tissues and repair damage.

Aprotinin, a protease enzyme inhibitor, inhibits the transformation of xanthine dehydrogenase into xanthine oxidase, thus preventing free oxygen radical formation.[20] While aprotinin inhibits the free oxygen radical formation, it can also inhibit bradykinin, kallidin, and other kinins.[12] Bradykinin, kallidin, and other kinins (known inflammatory mediators) have high activity as permeability factors.[21] Experimental studies have shown that aprotinin, in addition to its antiproteolytic membrane stabilizing property, decreases the release of lysosomal enzymes and increases intracellular adenine nucleotides.[22] Histological sections in the study described herein showed the protective effects of aprotinin in EU injury. To our knowledge, aprotinin has been used in EU injury for the first time here. Aprotinin reduced retinal thickness due to its protective effects on inflammatory reactions.

Melatonin is a pineal indolamine that is naturally located and normally synthesized in the retina by the action of hydroxyindole-*o*-methyltransferase, which is located at the photoreceptors; oxidative destruction in retinal tissue is therefore inhibited by melatonin.[4,13,14] Melatonin functions as a powerful ROS scavenger and antioxidant.[23] Melatonin is highly soluble in both lipid-based media and aqueous media (amphibolic) allowing it to reduce hydroxyl-mediated damage in both the lipid and aqueous subcellular compartments. This is an advantage because some other well-known antioxidants are exclusively lipid or water

soluble.[24] Yilmaz *et al*.[15] demonstrated the protective effects of melatonin on retinal oedema during ischemia–reperfusion in the guinea pig retina. We observed that retinas of the group treated with melatonin were protected from oedema and MDA formation (unpublished data). Therefore, the previous study[15] supports our results on melatonin supplementation in EU.

Vitamin C is a well known and important cytoprotective essential dietary nutrient required as a cofactor for many enzymes, the reduced form of the vitamin, ascorbic acid, is an especially effective antioxidant owing to its high electron-donating power and ready conversion back to the active reduced form.[8,25] The thickness of the retina and inner plexiform layer in the control group was significantly lower than in the vitamin C group, whereas the thickness in the retina and inner plexiform layer supplied with vitamin C was significantly lower than that in the EU group. We also observed that in the group treated with vitamin C the retina was protected from oedema and MDA formation (unpublished data). Our results are strongly supported by previous reports that vitamin C protects the retina against formation of EU.

ROS are active components of the phagocytic system of PMN.[1] In the event of neutrophils and macrophages becoming stimulated by pathogens, cytokines or other inflammation, mediators are liberated from the granula into the cytoplasm and play an important part in destroying phagocytosed materials.[26,27] Also, these cells are able to produce superoxide radicals and other oxidant species when activated by diverse stimuli.[7,19,26] The values of lipid peroxidation, mediated by neutrophils, depend on the severity of the inflammatory status.[27] For protecting against the oxidants that they produce, neutrophils acquire a high level of cell melatonin, vitamin C and aprotinin.[10,11]

In conclusion, three cytoprotective and free radical scavengers inhibited oedema formation in guinea pig retina accompanying EU. These results are important because retinal oedema is an important cause of the severe vision loss from uveitis. We concluded that aprotinin, melatonin and vitamin C may have useful effects on inflammatory oedema of the retina. However, the protective effect of vitamin C on EU was greater than that of aprotinin and melatonin.

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