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A short duration transient ischemia induces apoptosis in retinal layers: An experimental study in rabbits

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PURPOSE. *To investigate retinal cell apoptosis in an experimental transient, short duration ocular ischemia model.*
METHODS. *An experimental ischemia model, which simulates creating temporary high intraocular pressure to control intraocular bleeding during pars plana vitrectomy, was set up. Rabbits were randomly divided into three groups. Group 1 was the control group. In Group 2, intraocular pressure was increased to 97 mmHg for 5 minutes. In Group 3, intraocular pressure was increased to 97 mmHg for 10 minutes. After 24 hours, terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling assay was used to detect retinal apoptosis in rabbit eyes. Only nuclear staining in retinal cells was counted.*
RESULTS. *Groups with 5 minutes and 10 minutes of ischemia showed significantly higher amount of ganglion cell layer apoptosis when compared with the control group ($p<0.05$). Light microscopy and standard hematoxylin-eosin did not show any significant damage in the retina cells.*
CONCLUSIONS. *Apoptotic cell death in the retinal cell layers occurs in temporary ischemia-reperfusion as early as 5 and 10 minutes duration. (Eur J Ophthalmol 2005; 15: 233-8)*

KEY WORDS. *Arterial occlusion, Retinal cell apoptosis, Transient ischemia*

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INTRODUCTION

Retinal or ophthalmic artery occlusion causes acute cell death and severe irreversible visual loss (1-3). Retinal ischemia in experimental models can be induced by increasing the intraocular pressure (IOP) or by selective ligature of the ophthalmic artery (1-5). The relationship between the duration of the ischemia and cellular damage has been investigated by several studies (1, 2, 6-12). Cellular damage targets predominantly retinal ganglion cells (RGC). A second mechanism of ischemia-induced RGC death is a progressive event of apoptosis (13, 14). Apoptosis is a complex, programmed cell death initiated by specific signals that can be activated by caspases and characterized by a series of distinct morphologic and biochemical alterations (15).

In the present study, an experimental transient ocular ischemia model is described and retinal damage is studied. Our model simulates temporary IOP increase created during intraocular bleeding in pars plana vitrectomy.

MATERIALS AND METHODS

Experiments were conducted in strict accordance with the ARVO statement for the use of animals in

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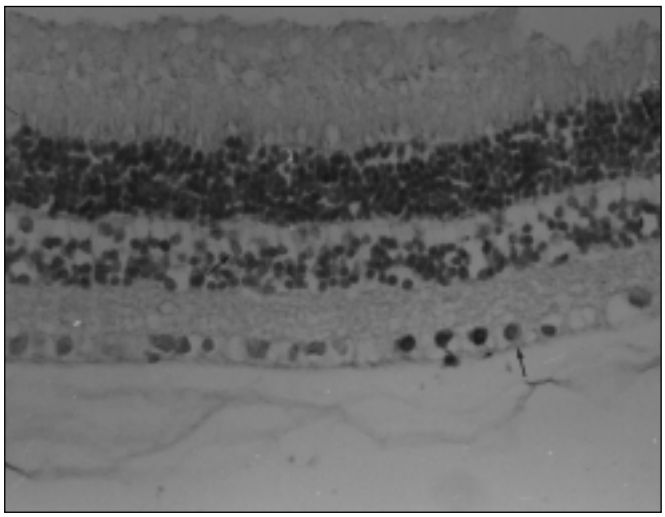

Fig. 1 - *TUNEL positive retinal cells in group with 5 minutes of ischemia (arrows indicate apoptotic cells).*

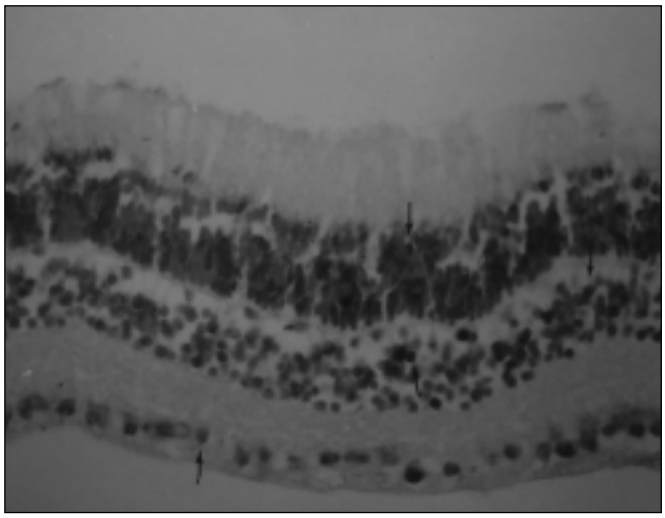

Fig. 2 - *TUNEL positive retinal cells in group with 10 minutes of ischemia (arrows indicate apoptotic cells).*

ophthalmic and vision research. Twenty-one New Zealand white rabbits (average weight 2.5 to 3.4 kg) were used in the study. Anesthesia was induced by an intramuscular injection of 25 mg/kg ketamine hydrochloride, 2.5 mg/kg xylazine, and topical proparacaine hydrochloride.

Vitreoretinal transient ischemia model

This experimental model was set up to simulate temporary high IOP created during pars plana vitrectomy to control intraocular bleeding, which causes transient interruption of retinal blood flow and results in transient ischemia. Animals were divided into three groups and each group consisted of seven eyes. Group 1 consisted of unwounded eyes and served as control. After cleaning the periocular and ocular surfaces with povidone iodide, a 4 mm infusion cannula was placed through pars plana sclerotomy.

A bottle of balanced salt solution (BSS Plus, Alcon Laboratories, TX) was attached to the infusion cannula. While elevating the bottle, retinal arteries were observed with indirect ophthalmoscopy to see the complete occlusion of the arteries and retinal whitening. At this point, the height of the bottle and the IOP were measured by Schiötz tonometer. The total IOP was increased to 97 mmHg, which was the sum of 105 cm water pressure (80 mmHg) and the mean 17 mmHg IOP and lasted for 5 and 10 minutes in Groups 2 and 3, respectively (Tab. I). At the end of the ischemic period the bottle was lowered to the eye level and retinal reperfusion was observed. Finally, the infusion cannula was removed and the sclerotomy sites and the conjunctiva were sutured with 7/0 Vicryl sutures. During recovery from anesthesia, the animals were placed in their cages, and a steroid–antibiotic ointment containing dexamethasone and tobramycin was applied. Rabbits were killed with 100 mg/kg intravenous pentobarbital injection 24 hours after the surgery in Groups 2 and 3. Rabbits in the control group were killed simultaneously and all eyes were enucleated. Eyes were evaluated histopathologically by light microscopy, immunohistochemical detection with terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL).

Histopathologic study

The eyes were fixed in 10% formaldehyde solution immediately after enucleation. The tissues were dehydrated and embedded in paraffin. The 5 µm sections were cut and stained with hematoxylin and eosin (H&E) for routine light microscopy.

TUNEL assay

Paraffin tissue blocks of all groups were cut to 4 µm thickness following routine deparaffinization and proteinase K (Applipere, Oncor 130202) was applied for 15 minutes. The tissues were stained according to the manufacturer's instructions with TUNEL assay by

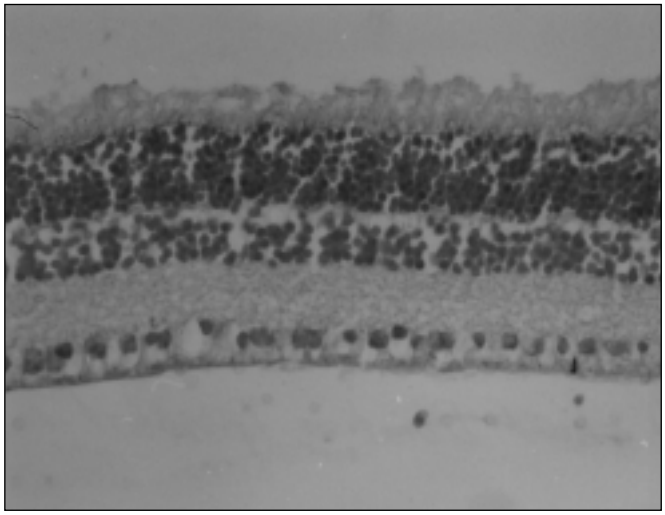

Fig. 3 - *TUNEL staining in the control group (no apoptotic cell).*

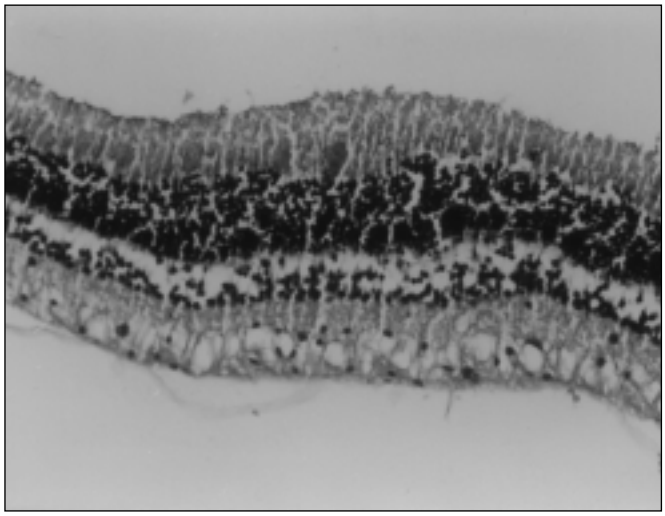

Fig. 4 - *No sign of cellular damage in any retinal layers in group with 10 minutes of ischemia by light microscopy (hematoxylin and eosin x400).*

using ApopTag plus peroxidase in situ apoptosis detection kits (S-7101, Oncor). Counterstaining of the specimen was made in 0.5% methyl green. Rat breast tissue was used as a positive control material. Four sections from each specimen were evaluated in the light microscopy at higher magnification fields (x400) and TUNEL+ cells were counted by the same observer. The number of stained cells was counted in 40 different fields of each specimen in ganglion cell layer, inner nuclear layer, and outer nuclear layer separately and the mean values were calculated for each group.

Statistical analyses

Analysis of variance was used to compare the results obtained in all groups and $p<0.05$ was considered significant. Data were analyzed using SPSS 9.0 for Windows (SPSS Inc.).

RESULTS

The number of apoptotic cells stained in the TUNEL assay after different periods of retinal ischemia is shown in Tables II, III, and IV. Groups 2 and 3 showed a significantly higher amount of ganglion cell layer apoptosis when compared with the control group ($p<0.05$). Also, inner nuclear layer and outer nuclear layer apoptosis were observed in Group 3 ($p<0.05$). TUNEL positive retinal layers were noted in Groups 2 and 3 (Figs. 1 and 2); however, in the control group (Group 1) no cells were stained (Fig. 3).

Light microscopy did not show any significant damage in retinal cells (Fig. 4). A few vacuolated changes without cystoid space and localized thickening in the nerve fiber layer were observed.

DISCUSSION

Numerous models have been created to induce transient retinal ischemia. One of the major methods is retinal or ophthalmic artery ligation with temporary sutures (5, 7, 11). The other is mechanical pressure on the eye to increase IOP with a number of techniques (3, 16). Our model simulates (or one-to-one corresponds with) increased IOP by elevation of the infusion bottle to control the intraoperative bleeding in pars plana vitrectomy. Finally, elevation of IOP with infusion fluid results in both vascular occlusion induced ischemia and mechanical injury to the retina. Our model has no similarity or analogy to isolated vascular occlusion such as clinical central retinal artery occlusion or ophthalmic artery occlusion.

Ischemic insult induced by an increased IOP over the systolic blood pressure produces damage to the inner retinal layers in rats (1-4). Clinically, increase of IOP by elevation of the infusion bottle to control the intraoperative bleeding results in pressure and vascular occlusion induced injury of the retina.

TABLE I - GROUPS

Group 1: Unwounded eyes: control group
Group 2: Intraocular pressure increased to 97 mmHg for 5 minutes
Group 3: Intraocular pressure increased to 97 mmHg for 10 minutes

TABLE II - RESULTS OF GANGLION CELL LAYER (GCL) APOPTOSIS IN THE CONTROL GROUP AND ISCHEMIA GROUPS

GCL groups	Apoptosis, mean cell count±SD	ANOVA test*	p value†
Group 1 (control)	1.14±0.46		
Group 2 (5 min)	7.86±2.04	0.03	<0.05
Group 3 (10 min)	10.57±7.46	0.003	<0.05

*Analysis of variance (ANOVA) test results show comparisons of Groups 2 and 3 with Group 1 (control group).
†$p < 0.05$ is significant

TABLE III - RESULTS OF INNER NUCLEAR LAYER (INL) APOPTOSIS IN THE CONTROL GROUP AND ISCHEMIA GROUPS

INL groups	Apoptosis, mean cell count±SD	ANOVA test*	p value†
Group 1 (control)	2.14±0.22		
Group 2 (5 min)	7.29±0.49	0.114	>0.05
Group 3 (10 min)	10.14±5.98	0.005	<0.05

*Analysis of variance (ANOVA) test results show comparisons of Group 2 and 3 with Group 1 (control group).
†$p < 0.05$ is significant

TABLE IV - RESULTS OF OUTER NUCLEAR LAYER (ONL) APOPTOSIS IN THE CONTROL GROUP AND ISCHEMIA GROUPS

ONL groups	Apoptosis, mean cell count±SD	ANOVA test*	p value†
Group 1 (control)	1.43±0.90		
Group 2 (5 min)	5.29±2.21	0.1	>0.05
Group 3 (10 min)	7.00±4.47	0.013	<0.05

Recent histopathologic studies have shown a decrease in RGC numbers after interruption of retinal blood flow in animal studies (4, 6-8). Histologic alterations to ischemia and reperfusion induced retinal damage are critically dependent on the duration of the ischemia. Ischemia for less than 60 minutes followed by reperfusion causes no gross histologic changes in the retina (7). Edema in the inner retinal plexiform layer was seen after 60 minutes of ischemic duration and neutrophil leukocyte infiltration, pyknotic nuclei, vacuolated spaces, and degenerative changes in the ganglion cells were observed after 90 minutes of ischemia followed by 24 hours of reperfusion (7). These findings are comparable to those of long-term ischemia of the eyes represented by central retinal artery occlusion (6). In our study, microscopy with H&E staining showed no sign of cellular damage in any of the retinal layers because of the short duration of the ischemic period. That was an expected finding in light of the previous studies (1, 2, 6-12). To our knowledge, there is no study describing apoptotic cell death in short duration ischemia reperfusions, such as 5–10 minutes. Quantification of the apoptosis process can be viewed on H&E sections with light microscopy. However, in light microscopy, only the end result of the process could be detected, missing the early apoptotic cells. The standard technique that can be applied to cell culture, frozen sections, and paraffin sections is known as TUNEL. There have been numerous publications using this method to demonstrate apoptosis (10, 14, 17). In addition, this technique can detect early-stage apoptosis in systems where chromatin condensation begins and strand breaks are fewer, even before the nucleus undergoes major morphologic changes (18, 19). Our study is the first in the literature to demonstrate the apoptotic cell death at the retinal cell layers without any significant damage that was seen by light microscopy and standard H&E staining in 5- to 10-minute durations of ischemia. RGC apoptosis was observed in both 5 minutes (Group 2) and 10 minutes (Group 3) of ischemia groups. However, apoptosis of inner and outer nuclear layer cells was detected only in 10 minutes (Group 3) of ischemia group. This finding may show that duration of ischemia longer than 5 minutes may damage not only RGC, but inner and outer nuclear layer cells as well, which is an important finding of our study.

We describe a different experimental transient ocular ischemia model that simulates creating temporary high IOP to control the intraocular bleeding during pars plana vitrectomy. This was associated with the apoptosis of various retinal layers demonstrated by TUNEL method in ocular ischemia models and found a significant apoptosis in retinal layers as early as 5 and 10 minutes of ischemia.

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