Local Hypothermia Protects the Retina from Ischemia

A Quantitative Study in the Rat

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We developed a quantitative histologic method for assessing injury in the rat retina due to transient ischemia. We used this technique to test the effectiveness of local hypothermia and allopurinol, an inhibitor of oxygen-free radical formation, in reducing ischemia/reperfusion injury in the rat retina. Retinal ischemia and reperfusion was produced by transient ligation of the optic nerve. Histologic evaluation by a masked observer was based on the average count of nonpyknotic nuclei in the inner nuclear layer of the retina from eight high power fields (X100) in one 5 µm thick sagittal section at or near the optic nerve. A sharp increase in tissue damage occurs between 90 and 120 min of ischemia. Ischemia for periods of 60 and 90 min produced mild damage while periods of 120 and 240 min produced severe damage. Hypothermia protected the retina significantly from 120 min of ischemic injury (P < 0.001 student t-test, compared to 120 min control), while allopurinol had no protective effect.

Ischemia plays a role in vasoocclusive and vasoproliferative diseases of the retina. Research on the pathogenesis and prevention of ischemic injury in the retina has been hampered by the lack of a quantitative method to assess ischemic damage. Previous studies have used semiquantitative and qualitative methods of evaluation such as electroretinography and fluorescein angiography. We have developed a quantitative histologic technique to assess ischemic injury in the rat retina.

Ischemia leads to the depletion of a cell's ability to produce a sufficient quantity of ATP to maintain normal cellular function and ion homeostasis. The period of ischemia a tissue can withstand before irreversible damage occurs is known as the tolerance time. If the ischemia continues for a sufficient period of time, the tolerance time of the tissue is exceeded and cell death and tissue necrosis will occur. Reperfusion of ischemic tissue before irreversible damage has occurred may allow tissue recovery. Hypothermia has been shown and used to protect tissues such as the heart and brain from ischemic injury by extending the tolerance time to ischemia. The effectiveness of hypothermia in protecting the retina from ischemic injury has not previously been investigated.

Upon reperfusion, the ischemic tissue may suffer further damage secondary to oxygen-free radicals formed during the reperfusion. Oxygen-free radicals have been implicated in ischemia/reperfusion injury in tissues such as intestine, heart, and brain. Allopurinol inhibits oxygen-free radical formation, and can reduce injury in the reperfusion of ischemic tissues such as intestine, liver, stomach and heart. The possible role of oxygen-free radicals in ischemia/reperfusion injury in the retina has not previously been investigated.

We used our quantitative method to assess the effectiveness of local hypothermia and allopurinol at reducing transient retinal ischemic injury.

Materials and Methods

We created retinal ischemia and reperfusion in the rat using a technique that has been described previously. Rats weighing 225–500 g were anesthetized with ketamine HCl 15 mg/100 g injected intramuscularly followed by an intraperitoneal injection of 0.4 mg/100 g of xylazine. The fundus was examined with a Zeiss operating microscope and a lateral canthotomy and peritomy were performed. The optic nerve was exposed with careful blunt dissection and ligated with 4.0 silk suture. After the ligation, ischemia was verified by funduscopy examination with the operating microscope, which revealed absence of blood flow in all visible retinal vessels. After the specified time of ischemia, nonperfusion was again verified fundu-
scopically and the ligature was carefully removed. Reperfusion was verified by funduscopic exam with the operating microscope, which revealed blood flow in all visible vessels. If full reperfusion was not achieved within 15 min of suture release, the animal was removed from the study. The allopurinol treated rats were fed allopurinol 30 mg/100 g body weight per day for 2 days prior to undergoing the ischemia/reperfusion procedure. In one group of rats, the ischemic eye was cooled with an external icepack which was in place within 5 min of the initiation of the ischemia and remained in place until approximately 5 min before ligature release.

After the specified period of reperfusion (usually 48 hr) the rats were anesthetized as before and the eyes examined with the operating microscope. The rats were then killed with an intracardiac injection of sodium pentobarbital and the eyes were enucleated. The eyes were immediately fixed with Zenker's solution. After fixation, the eyes were embedded in paraffin and 5 μm thick sagittal sections through or near the optic nerve were obtained and stained with hematoxylin and eosin. The retinas were then evaluated by a masked observer.

The evaluation was based upon the number of nonpyknotic nuclei present in the inner nuclear layer of the retina. Pyknotic nuclei were distinguished by the condensation of chromatin with loss of normal chromatin pattern (Fig. 1C). The nonpyknotic nuclei of cells in the inner nuclear layer were counted from eight high power fields (×100) in one sagittal section at or near the optic nerve in each eye. The eight fields consisted of four from the posterior pole and four from the peripheral retina, two from each side at a fixed distance of five fields from the ora serrata. The average count of nonpyknotic nuclei per high power field in each eye was based on the average of the mean and posterior pole counts. The sections were also evaluated for edematous changes, retinal detachments, and hemorrhages.

All animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

**Results**

Normal control (0 min of ischemia)—a total of 31 unoperated normal eyes were evaluated. The mean non-pyknotic nuclei count per high power field ± 1 standard deviation was: 128 ± 12. There was no clinical or histologic evidence of edematous changes, retinal detachment, or retinal hemorrhage. Figure 1A represents the histologic appearance of a normal rat retina.

Ten eyes underwent 60 min of ischemia and 48 hr of reperfusion. The mean nonpyknotic nuclei count was 111 ± 28. Compared to 0 min of ischemia, there was no significant statistical difference between the average nuclei counts with $P = 0.009$ (unpaired student t-test). Four out of the 10 eyes had histologic evidence of vacuolated spaces consistent with edematous changes, three of the eyes revealed histologic evidence of a shallow retinal detachment confined to the retina surrounding the optic nerve head, and none of the eyes demonstrated any evidence of retinal hemorrhage.

Eight eyes underwent 90 min of ischemia and 48 hr of reperfusion. The mean nonpyknotic nuclei count was 105 ± 22. Compared to 60 min of ischemia, there was no significant statistical difference between the average nuclei counts with $P = 0.624$ (unpaired student t-test). Four out of the eight eyes had histologic evidence of vacuolated spaces consistent with edematous changes, and none of the eyes demonstrated any evidence of retinal detachment or hemorrhage. Figure 1B demonstrates a rat retina which has undergone 90 min of ischemia followed by 48 hr of reperfusion.

Fourteen eyes underwent 120 min of ischemia and 48 hours of reperfusion. The mean nonpyknotic nuclei count was 53 ± 17. Compared to 90 min of ischemia, there was a significant statistical difference between the average nuclei counts with $P < 0.001$ (unpaired student t-test). Thirteen of the eyes had histologic evidence of vacuolated spaces consistent with edematous changes, three of the eyes revealed histologic evidence of a shallow retinal detachment, and two of the eyes had evidence of retinal hemorrhage. Figure 1C demonstrates a rat retina which has undergone 120 min of ischemia followed by 48 hr of reperfusion.

Ten eyes underwent 240 min of ischemia and 48 hr of reperfusion. The mean nonpyknotic nuclei count was 24 ± 9. Compared to 120 min of ischemia, there was a significant statistical difference between the average nuclei counts with $P < 0.001$ (unpaired student t-test). All 10 eyes had histologic evidence of vacuolated spaces consistent with edematous changes, and six of the eyes revealed histologic evidence of a small retinal detachment and retinal hemorrhage. Figure 1D demonstrates a rat retina which has undergone 240 min of ischemia followed by 48 hr of reperfusion.

In the rats treated with allopurinol, six eyes underwent 120 min of ischemia and 48 hr of reperfusion. The mean nonpyknotic nuclei count was 51 ± 9. Compared to 120 min of ischemia without treatment, there was no significant statistical difference between the average nuclei counts with $P = 0.783$ (unpaired...
Fig. 1. Effect of ischemia on rat retina without treatment. (A) Normal. (B) After 90 min of ischemia and 48 hr of reperfusion. (C) After 120 min of ischemia and 48 hr of reperfusion with numerous number of pyknotic nuclei present. (D) After 240 min of ischemia and 48 hr of reperfusion with numerous number of pyknotic nuclei and mitotic figures present. Hematoxylin and eosin (original magnification ×520).

Two groups of rats underwent 120 min of ischemia followed by 7 days of reperfusion. One group of control rats and a group treated with allopurinol underwent 120 min of ischemia followed by 7 days of reperfusion. The control group consisted of 7 eyes. The mean nonpyknotic nuclei count was 87 ± 34. The allopurinol-treated group consisted of three eyes. The mean nonpyknotic nuclei count was 83 ± 27. Compared to 120 min of ischemia without treatment with allopurinol, there was no significant statistical difference between the average counts with \( P = 0.872 \) (unpaired student t-test). All of the eyes had histologic evidence of vacuolated spaces consistent with edematous changes in the retina, and none of the eyes demonstrated evidence of retinal detachment or hemorrhage.

Discussion

The results demonstrate a sharp decrease in the number of non-pyknotic nuclei between 90 and 120 min of ischemia.
min of ischemia. Rats subjected to 90 min or less of ischemia demonstrated mostly mild or no tissue damage, while ischemia for periods of 120 min or more demonstrated severe damage. Figure 1B demonstrates a rat subjected to 90 min of retinal ischemia with a normal number of nuclei in the inner nuclear layer and no evidence of tissue damage. Figure 1C represents the severe tissue damage seen typically in rats subjected to 120 min of retinal ischemia. Several rats subjected to 90 min or less of retinal ischemia demonstrated some tissue damage. This may represent a variable biological susceptibility to ischemia or injury caused by the occlusion procedure.

These results parallel clinical and other experimental evidence of the retina's tolerance time to ischemia, time of ischemia before irreversible damage occurs.

Observation of clinical cases of central retinal artery occlusion have led to the conclusion that the human and nonhuman primate retina can withstand 90 but not greater than 120 min of transient ischemia. Experimental transient retinal ischemia in rhesus monkeys demonstrated that periods of ischemia up to 98 min produced no significant irreversible damage.2

Our results demonstrated that the application of local hypothermia with an external icepack extended retinal tolerance time to ischemia and protected the retina from 120 min of ischemia. All the rats subjected to 120 min of ischemia with the application of local hypothermia had nonpyknotic inner nuclear layer nuclei counts that were normal or near normal and greater than twice the number of nonpyknotic nuclei in the control group. The difference in nuclei counts between the cooled eyes and the control eyes with the same ischemic period was highly statistically significant at \( P < 0.001 \) (unpaired student t-test). Although the mechanism for how hypothermia protects the retina from ischemia is not certain, it is likely that the local hypothermia causes a decrease in tissue temperature that decreases the rate of chemical reactions.

Ischemia alone can cause tissue damage, but ischemic tissue may suffer further damage when reperfused. Oxygen-free radicals have been implicated in the injury associated with reperfusion of ischemic tissue. It has been proposed that a major source of oxygen-free radicals in ischemic tissue is xanthine oxidase.10 Xanthine oxidase is formed in ischemic tissue from the widely distributed enzyme xanthine dehydrogenase by a protease activated by the increasing intracellular calcium concentration due to the low energy state of the cell. Upon reoxygenation of the ischemic tissue, xanthine oxidase can catalyze the reaction of molecular oxygen and hypoxanthine, a low
energy breakdown product of ATP, to form the superoxide radical and hydrogen peroxide. Allopurinol is an inhibitor of xanthine oxidase and has been shown to be effective in reducing ischemia/reperfusion injury in tissues such as intestine and heart.

Our results demonstrated that there was no statistically significant difference between the cell counts at 48 hr and 7 days in the rats treated with allopurinol and the control group after 120 min of retinal ischemia. Pretreatment with allopurinol did not protect the retina from ischemia and reperfusion injury. These results suggest that the production of oxygen-free radicals by xanthine oxidase does not play a major role in the injury caused by transient ischemia/reperfusion of the rat retina. Because there are alternate pathways for the production of oxygen-free radicals, such as granulocyte NADPH oxidase, which allopurinol would not affect, our experiments have not ruled out the possible role of oxygen-free radicals in ischemia/reperfusion injury in the retina.

In the eyes that had 120 min of ischemia and 7 days of reperfusion, the nuclei counts in these eyes were higher than the comparable counts after 48 hr of reperfusion. The reason for the higher nuclei counts may lie in the mitotic activity seen after ischemia and reperfusion in the retina, as was seen in this study (Fig. 1D) and previously reported.

In summary, we have developed a method to quantify the damage caused by transient ischemia in the rat retina. A sharp increase in irreversible tissue damage occurs between 90 and 120 min of ischemia. Ischemia for periods of 90 min or less produced mild tissue damage while periods of 120 min or more produced severe damage. This technique of assessing transient ischemia can be used to explore the pathogenesis of ischemic damage and test agents that may reduce ischemic injury. We used this method to demonstrate that allopurinol, an inhibitor of xanthine oxidase, did not reduce ischemia/reperfusion injury in the rat retina, but local hypothermia did protect the rat retina from 120 min of ischemia.

Cooling the eye protects the retina from tissue damage in temporary retinal ischemia in the rat. Further research is needed to determine whether the retina of a larger eye, such as the human eye, can be protected similarly from ischemic injury.

Key words: retina, ischemia, hypothermia, allopurinol, oxygen-free radicals

References