INTRODUCTION

Glaucoma is a chronic and progressive disease that is manifested by death of retinal ganglion cells (RGCs), loss of axons, and an irreversible loss of vision. Clinical observations indicate that glaucoma patients have late subjective visual symptoms or loss of central visual acuity. Experimental glaucoma studies in animal models have proven that the RGCs in the peripheral retina are more susceptible than those in the central retina. These data suggest that retinal...
ganglion cell (RGC) loss in the glaucomatous retina is affected by the site in which RGCs are located. In efforts to determine the mechanism(s) leading to selective loss of RGCs, numerous studies have been designed to detect the correlation between selective loss of RGCs and RGCs’ autologous features in the glaucomatous retina. First, based on the diameter of axonal fibers and size of ganglion cell soma in both normal and glaucomatous eyes, some investigators claim that larger RGCs are more susceptible than smaller ones in the glaucomatous retina. However, controversial data from experimentally induced glaucoma in monkeys argue that there is no apparent selective loss of larger RGCs. Furthermore, data from psychophysical tests on glaucoma patients do not support a preferential loss of larger RGCs in glaucoma. Second, target-derived stimulation by neurotrophic factors (NTs), such as brain-derived neurotrophic factor and ciliary neurotrophic factor, have been shown to promote neuronal survival. In an experimental glaucoma model, it was shown that retrograde axonal transport of NTs and their receptors is blocked. Furthermore, exogenous NTs are able to prevent the loss of RGCs in the glaucomatous retina. Thus, some researchers proposed that the differential effects of NT receptors on RGCs leads to the selective loss of these cells. However, this latter concept is doubtful since no apparent correlation between Trk receptor (the high affinity receptor of NTs) expression and RGC survival was found either for the optic nerve trans-section model or the chronic high intraocular pressure model (CHIP). Third, after Dreyer et al. first reported that glutamate levels in the vitreous body of humans and monkeys with glaucoma were elevated, much attention has been paid to (i) glutamate excitotoxicity in the glaucomatous retina and (ii) the effects of differential expression of glutamate receptors on the RGC susceptibility. Unfortunately, the disparity in expression of glutamate receptors is not closely related to the selective loss of RGCs in glaucoma. Taken together, these data suggest that the correlation between selective loss of RGCs and RGCs’ own characteristics in the glaucomatous retina is uncertain, seriously hindering early detection, diagnosis, treatment, and possible prevention of glaucoma.

A number of environmental factors are vital to the development and survival of RGCs. Of these, blood supply is one of the most important supportive factors. In the vast majority of glaucoma patients, the blood supply to the retina is substantially impaired. For example, reduced blood flow to the retina and optic nerve head of glaucoma patients has been detected (using angiography and the Heidelberg retina flowmeter). In addition, narrowed arteries and dilated veins have been observed in glaucoma patients (using the retinal vessel analyzer). Moreover, increased diameters of retinal veins of glaucoma patients are significantly less than those of controls, as shown in the flicker stimulation test. Furthermore, stimulation of flicker caused a 39% increase in blood flow to the optic nerve head of persons without glaucoma, in contrast to the 10.4% increase in early glaucoma patients. These data indicate that there is a deficiency of blood supply to the glaucomatous retina. A large number of studies of glaucoma patients have shown that there is a reduction in peripheral blood flow as well as reduced blood flow to tissues other than retina, including optic nerve, choroid, and retrobulbar tissue. Reduction of local blood supply, via application of endothelin-1 (causing vessel constriction) to the perineural region of the anterior optic nerve head, induced glaucoma-like damage in animals with normal intraocular pressures (IOPs). Pharmacological improvement of circulation, e.g., by using carbonic anhydrase inhibitors or calcium channel blockers, was helpful for reversing visual field defects in glaucoma patients. The disruption of retinal blood supply appears, therefore, to be at least partially responsible for the loss of RGCs in the glaucomatous retina. However, these previous researches focused only on the whole retina. It remains to be seen whether changes of local blood supply to the glaucomatous retina differ for the central, middle, and peripheral portions of the entire retina. It is also unclear whether heterogeneous deficits of local blood supply to the glaucomatous retina are related to the selective loss of RGCs. In the present study, we used the acute high intraocular pressure (AHIP) model to partially mimic the pathology of clinical glaucoma. We examined the blood supply to the central, middle, and peripheral portions of the retina at different time points following AHIP using gelatin-ink perfusion and injection of fluorescent microspheres. Moreover, we investigated the relationship between regional changes of blood supply and loss of RGCs in the central, middle, and peripheral regions of the glaucomatous retina.

MATERIALS AND METHODS

Animals and Grouping

One hundred and sixty-two adult Sprague-Dawley rats (200–250 g) were used in this study (purchased from Central South University, P.R. China). All animals were housed under controlled environmental conditions on a 12h light/dark cycle with ad libitum access to food and water. All protocols were approved by the local animal ethics committee, and were in conformance with the Chinese government animal protection and management law, and the guidelines for animal experiments of Central South University.
Rats were randomly divided into gelatin-ink \((n = 54)\), microsphere \((n = 54)\), and fluorogold \((n = 54)\) groups. Rats in the gelatin-ink group were infused with the gelatin-ink mixture, as previously described,\(^1\) injected with fluorescent microspheres from the vena femoralis in the microsphere group, and labeled with fluorogold at the bilateral superior colliculi in the fluorogold group. Each group was further subdivided into control \((n = 6)\), sham operation \((n = 6)\), and experimental \((n = 42)\) subgroups. Rats in experimental subgroups were subjected to AHIP and then maintained for 3, 6, and 12 h, and 1, 3, 7, and 14 d \((n = 6\) at each time point in each subgroup).

**AHIP Model**

The animal model was prepared following the procedure described by Chen et al.\(^2\) Animals were anesthetized with 2% pentobarbital (40 mg/kg). A drop of chloramphenicol was administered to each conjunctival sac. Two 30-gauge needles, connected to the installation instrument filled with normal saline, were inserted into the anterior chamber. Then, the IOP was elevated to 110 mmHg, maintained for 60 min, and then slowly decreased to normal. In the sham operation subgroups, the needle was inserted into the anterior chamber without elevating the IOP, and the rats were maintained for 1 d.

**Gelatin-Ink Infusion**

The gelatin-ink infusion method employed was as described previously.\(^3\) Briefly, the systolic blood pressure was measured using the tail cuff method with an electrosphygmomanometer (BL-New Century, Chengdu, China). Then, rats were anesthetized, and the thoracic cavity was opened and the right atrium cut to ensure outflow. Blood washout was achieved with a left ventricular puncture using a 12-gauge needle and a constant pressure perfusion assembly. The washout medium was 0.9% saline at 37°C. The abdominal aorta was ligated just under the diaphragm to enhance the perfusion effect above the diaphragm. When the fluid flowing out of the right atrium was clear, rats were infused with 20 ml of 37°C ink containing 3% gelatin at 140% rat mean arterial pressure (MAP)—the MAP for all rats is 113 ±7 mmHg—and then with 20 ml of 37°C ink plus 5% gelatin at 180% rat MAP. The superior vena was ligated after the ink was observed exiting from the right atrium, and the thoracic aorta was ligated at the end of the infusion. Successful perfusion was indicated by marked blackening of the eyes, snout, and ears. In order to coagulate the gelatin-ink mixture, rats were placed at 4°C for 40 min, eyes were then enucleated, and the retina dissected, post-fixed in 4% paraformaldehyde for 4 h at 4°C, and immersed in 30% sucrose. Finally, whole-mounts of retina were dried, dehydrated, and cover-slipped.

In order to demonstrate local blood supply, we calculated the percent microvessel area of the total tissue area by using methods described by Quigley et al.\(^4\) Briefly, retinas were divided into four quadrants, each containing three regions of 430 × 320 µm\(^2\) each. These were 1/6, 3/6, and 5/6 of the retinal radius away from the optic nerve head—referred to as central, middle, and peripheral retina, respectively.\(^5\) Thus, 12 regions for each retina were counted. The total area of the retina and the area occupied by microvessels lumens from any one region were measured using a Motic Images Advanced 3.2 system.\(^6\) The ratio of the two areas was calculated and referred to as the percent microvessel area. Central retinal vessels and major branches were excluded from measurements of the microvascular area. The percent microvessel areas of the central, middle, and peripheral retina were calculated as the average value of the corresponding region in four quadrants. Because the percent microvessel areas of the central, middle, and peripheral retina were different in the normal retina, and in order to compare changes in local blood supply, we standardized the increased percent microvessel area by comparisons to the control group at each time point. For example, the increased percent microvessel area of the central retina in the experimental subgroup at 3 h was calculated as the percent microvessel area of the central retina in the experimental subgroup at 3 h minus the percent microvessel area of the central retina of the control group divided by the percent microvessel area of the central retina of the control group multiplied by 100%.

**Fluorescence Microsphere Injection**

Following the procedures described by Ben-nun et al.,\(^7\) animals were anesthetized with 2% pentobarbital (40 mg/kg). The femoral vein was cannulated, and 250 U (1,000 U/ml) of heparin sulfate was injected. Fluorescent polystyrene microspheres (Duke Scientific, Palo Alto, California, USA), 0.5 µm in diameter, were perfused at 0.1 ml/min for 5 min (microsphere concentration was 1.4 ×10\(^8\)/ml). Loops were placed around the optic nerves of both eyes, and both ocular circulations were clamped abruptly to cause instant arterial occlusion. Using this technique, blood flow is assumed to be arrested instantly and blood components remain in the same location as they were at the time of occlusion. Eyes were enucleated and placed in 4% paraformaldehyde. Then, 15 min later, anterior segments and lenses were

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removed, and the retinas washed directly via a gentle flow of 4% paraformaldehyde. During this time, retinas were being dissected free from the underlying choroid. Retinas were then whole-mounted and cover-slipped. According to the principle of data collection mentioned above, microspheres in the central, middle, and peripheral retina were photographed and counted by the methods described by Wang and Ahmed. Microsphere density was calculated in the central, middle, and peripheral portions, as described in “Gelatin-ink infusion” method. Because the microsphere density of the central, middle, and peripheral retina were different from those of the normal retina, and in order to compare changes in local blood supply, we also standardized increased microsphere density by comparisons to the control group at each time point using methods described in the “Gelatin-ink infusion” method.

**Fluorogold Retrograde Labeling**

The fluorogold labeling method was as described previously. Each rat was deeply anesthetized peritoneally using 2% pentobarbital (40 mg/kg). Bilateral superior colliculi were exposed and the pia overlying the superior colliculus removed. A piece of gelfoam, soaked in a solution of 5% fluorogold (Fluorochrome, LLC) and 10% dimethylsulphoxide in saline, was placed on the surface of the superior colliculus and the skin sutured. Seven days later, rats were subjected to the various experimental treatments and sacrificed at the specified time points. Whole-mounts of retinas were observed using fluorescence microscopy. Fluorogold-labeled RGCs in the central, middle, and peripheral portions of the retina were photographed and manually counted by a person totally blind to the experimental design. RGCs were identified by fluorogold labeling and shape (round or oval). RGC densities in the central, middle, and peripheral retina were calculated. Because RGC densities of the central, middle, and peripheral retina were different from those of the normal retina, and in order to compare the loss degree of RGCs after AHIP, we calculated the percent loss of RGCs. For example, percent loss of RGCs in the central retina of the experimental subgroup at 3h was calculated as RGC density in the central retina of the control group minus RGC density in the central retina of the experimental subgroup at 3h divided by RGC density in the central retina of the control group multiplied by 100%.

**Statistical Analysis**

All data are presented as mean ± standard deviation (± SD). One-way analysis of variance (ANOVA) and the Kruskal Wallish test were used for means comparisons. In addition, the percentage of RGC loss was correlated to the increased percent microvessel area and microsphere density by linear-regression analysis. A standardized regression coefficient was further calculated by multiplying the regression coefficient; p-values < 0.05 were considered statistically significant.

**RESULTS**

**Changes of Local Blood Supply Following AHIP**

The percent microvessel area in a specific region is generally used to reflect local blood supply. In the present study, retinal vessels were visualized using our previously published gelatin-ink infusion method. Both the percent microvessel area and the increased percent microvessel area relative to control were calculated. Each was calculated to reflect (i) regional blood supply and (ii) changes in regional blood supply. Following AHIP, we show that there was a greater proportion of microvessel area in the central (Figure 1A) and middle than in the peripheral (Figure 1B) portions of the normal retina, with increases at 3 and 6h (Figures 1C and 1D), decreases at 12h, and 1 d, and partial recovery by 14 d. The increased percent microvessel area differed from central to peripheral retina at all time points (Figure 2A), with smaller increases in the peripheral retina than either in the central or middle retina (Figure 2A). At 3h following AHIP, the percent increase in the peripheral retina was -1.2 ± 0.4%, significantly lower than the 7.1 ± 0.4% calculated for the central retina and 8.6 ± 0.3%.
Differential Changes of Local Blood Supply

429

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for the middle retina (p < 0.05) (Figure 2A). At 6 h, the percent increase for the peripheral retina was 4.7 ± 1.5%, which was also lower than the 12.8 ± 3.3% calculated for the central retina and the 25.8 ± 0.4% for the middle retina (p < 0.05) (Figure 2A). These data suggest that the blood supply to the central and middle retina increased at 3 and 6 h, and then decreased at 12 h to 14 d following AHIP. The increased blood supply to peripheral retina was less than that to either the central or middle retina at all time points following AHIP.

The use of intravascular microspheres as deposition markers for detecting regional blood supply is a commonly used blood supply measurement technique in a wide range of research fields. In order to validate the changes of local blood supply that were shown by the gelatin-ink infusion method, we examined the changes of blood supply to the regional portions of the retina by examining the percent of microsphere density. Changes in local blood supply shown by microspheres injection were consistent with gelatin-ink infusion method data (Figure 2B).

Differential Loss of RGCs Following AHIP

Fluorogold retrograde labeling is commonly used for detecting RGCs. Using this method, we found there to be a progressive loss of RGCs in the central, middle, and peripheral portions of the retina diminished as reperfusion times increased. However, there was a greater decrease in the number of RGCs in the peripheral retina versus the central or middle retina (p < 0.05). *p < 0.05 versus the central retina at the same time point.

FIGURE 2 Changes in local blood supply in rat retina following AHIP. Gelatin-ink perfusion (A) and injection of fluorescent microsphere (B). In A, the increased percent retinal microvessel area (PRMA) at each survival time point compared to the control group was used to represent the change of local blood supply. In B, the number of microspheres indicate the local blood supply change at each specified time point. These two methods showed similar changes in local blood supply in the rat retina, with progression of the reperfusion time following AHIP. The increased percentages of local blood supply to the peripheral retina were always lower than those to the central and middle retina at each time point during the whole reperfusion. *p < 0.05 versus the peripheral retina at the same time point; #p < 0.05 versus central retina at the same time point.

FIGURE 3 Compared to the central (A) and peripheral (B) portions of normal retina, patchy loss of fluorogold-labeled RGCs is observed in the central (C) and peripheral (D) retina at 6 h after AHIP. Moreover, the RGC loss in the peripheral (D) retina at 6 h after AHIP is more obvious, relative to the central portion (C). Bar = 100 μm.

FIGURE 4 Loss of RGCs in different portions of rat retina during the reperfusion following AHIP. The number of fluorogold-labeled RGCs in the central, middle, and peripheral portions of the retina diminished as reperfusion times increased. However, there was a greater decrease in the number of RGCs in the peripheral retina versus the central or middle retina (p < 0.05). *p < 0.05 versus the central retina at the same time point.

AB
CD

* A
** C
# D

* A
** C
# D

A
B
C
D

% RGCs lost
0.0
20.0
40.0
60.0
80.0
100.0
120.0
3h 6h 12h 1d 3d 7d 14d
Reperfusion time

Peripheral
Middle
Central

120.0
100.0
80.0
60.0
40.0
20.0
0.0
3h 6h 12h 1d 3d 7d 14d
Reperfusion time

Peripheral
Middle
Central

20.0
40.0
60.0
80.0
100.0
120.0
3h 6h 12h 1d 3d 7d 14d
Reperfusion time

Peripheral
Middle
Central

* A
** C
# D

* A
** C
# D

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central retina, 93.2% in the middle retina, and 95.3% in the peripheral retina ($p > 0.05$) (Figure 4).

**Correlation between Increased Local Retinal Blood Supply and RGC Loss Following AHIP**

Similar changes in local retinal blood supply following AHIP are detected by the gelatin-ink infusion and microsphere injection methods. Linear correlation analysis showed positive correlations between the two methods ($r = 0.82, p = 0.000, r^2 = 0.76$), suggesting that the two methods share the same sensitivity for detecting blood supply in the retinal regions (Figure 5). In order to determine whether the changes in local retinal blood supply impacted RGC survival following AHIP, the loss of RGCs in each region was correlated to changes in local blood supply. There was a significantly negative correlation between the percent loss of RGCs and increased local blood supply to the whole retina following AHIP ($r = -0.57, p = 0.007, r^2 = 0.44$ by the gelatin-ink infusion method, and $r = -0.72, p = 0.000, r^2 = 0.51$ by the microsphere injection method) (Figure 6). Since local blood supply showed maximal changes during the first day following AHIP, a correlation for the first day was calculated: $r = -0.67, p = 0.016, r^2 = 0.45$ (gelatin-ink infusion method) and $r = -0.74, p = 0.006, r^2 = 0.55$ (microsphere injection method) (Figure 7). For the peripheral retina, the degree of correlation was $r = -0.80, p = 0.030, r = 0.64$ (gelatin-ink infusion method) and $r = -0.91, p = 0.005, r^2 = 0.83$ (microsphere injection method) (Figure 8). For the central and middle retina, the correlations were not statistically significant ($p > 0.05$) (Figure 8).

**DISCUSSION**

Previous investigations have shown that disruption of blood supply to the whole retina is at least partially...
Differential Changes of Local Blood Supply

responsible for RGC loss in the glaucomatous retina.15,16

By comparing changes in the local blood supply to rat retina—from central to peripheral retina following AHIP—we demonstrate, for the first time, that RGC loss is negatively correlated to increases in the blood supply to local retinal regions following AHIP.

Early changes in blood supply to the retina have been widely studied in acute ischemia-reperfusion models. For example, Chu et al.30 found that hyperemia existed in rat retinal blood vessels in the early stages following AHIP. Hirose et al.31 induced transient retinal ischemia for 60 min by ligation of the optic sheath, and found that the major retinal arteries constricted and the major retinal veins dilated during the first 72 h of reperfusion. Consistent with these studies, our results demonstrated that the blood supplies to the central, middle, and peripheral retina increased at 3 and 6 h following AHIP. Blood supplies to the central, middle, and peripheral retina decreased at 12 h, and 1 and 3 d. Our data further indicate that the increase in blood supply to the peripheral retina was lower than to either the central or middle retina. These data suggest that changes in local retinal blood supply are unequal in central, middle, and peripheral retina following AHIP. Furthermore, these

\[
FG = 43.69 + -1.15 \times \text{ink} \\
\text{R-Square} = 0.45
\]

\[
FG = 81.88 + -0.33 \times \text{ink} \\
\text{R-Square} = 0.02
\]

\[
FG = 53.28 + -0.27 \times \text{microsphere} \\
\text{R-Square} = 0.55
\]

\[
FG = 86.43 + 0.06 \times \text{microsphere} \\
\text{R-Square} = 0.01
\]

FIGURE 7 Correlation between loss of RGCs and changes in local blood supply at various stages of reperfusion. There was a significantly negative correlation between the PRMA and decreased RGC density in rat retina during the first day of reperfusion following AHIP (A), \( r = -0.67, r^2 = 0.45, p = 0.007 \). However, there was no significant correlation after 1 d of reperfusion (B), \( r = -0.17, p = 0.668 \), PRMA: percent retinal microvessel area shown by gelatin-ink perfusion. Similar negative correlations exist between the increased microsphere density and decreased RGC density in rat retina at early stages (C), \( r = -0.74, r^2 = 0.55, p = 0.000 \). No significant correlation was detected at late stages (D). \( r = 0.10, p = 0.798 \).

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changes are similar to the pattern of changes in local blood supply to the retina of glaucomatous patients.\textsuperscript{32} Duijm et al.\textsuperscript{32} assessed arteriovenous passage times in retinal vessels of glaucomatous and normal eyes by digitized scanning laser fluorescein angiography. They found that the difference in arteriovenous passage times of peripheral retina between glaucomatous and normal subjects was considerably larger than for central retina. Although the blood supply to the peripheral retina was impacted more perceptibly than

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8.png}
\caption{Correlation between loss RGCs and changes in local blood supply to central, middle, and peripheral retina. There was a significantly negative correlation between the increased PRMA and decreased RGC density in the peripheral retina after AHIP (A). $r = -0.80$, $r^2 = 0.64$, $p = 0.030$. PRMA: percent retinal microvessel area shown by gelatin-ink perfusion. A similar negative correlation also exists between increased microsphere density and decreased RGC density in peripheral retina (D). $r = -0.91$, $r^2 = 0.83$, $p = 0.005$. However, there was no significant correlation between the middle (B, E) and central (C, F) portions of retina ($p > 0.05$).}
\end{figure}
that to the central retina, the mechanisms underlying such differences remain unknown.

Fluorogold retrograde labeling could be regarded as the classic method for investigating RGC survival. In our study, RGCs were pre-labeled with fluorogold before initiating AHIP. This process allowed us to identify the presence of RGCs at various time points following AHIP. Our results show that there was a patchy loss of RGCs after exposure to AHIP, a finding similar to that found in the CHIP model. Additionally, after CHIP, the rates of loss of RGCs in the central and middle retina were similar. These data all refer to the selective loss of RGCs following AHIP. Yet, the loss of RGCs in the peripheral retina was clearly greater than in either the central or middle retina during the first 3 days following AHIP, especially during the first 24h. Peripheral RGC susceptibility was also observed in the CHIP model. Additionally, after CHIP, the rates of loss of RGCs in the central and middle retina were similar. These data all refer to the selective loss of RGCs in experimental glaucoma. In order to elucidate the mechanism(s) leading to such selective loss of RGCs, we used linear-regression analysis to study the effects of changes in local retinal blood supply on RGC loss following AHIP. Our results showed that the percentage loss of RGCs is negatively correlated to increased local blood supply, as shown by gelatin-ink infusion and microsphere injection methods (r² = 0.44 and 0.51, respectively). During the first 24h, the local retinal blood supply displayed the maximal change, with a higher degree of correlation between loss of RGCs and increased blood supply. For the peripheral retina, where there was less of an increase in blood supply than to either the central or middle retina, the degrees of correlation were much higher than for the central or middle retina. These data suggest that changes in local retinal blood supply impact RGC survival following AHIP. However, it is unclear whether or not similar correlations exist in the CHIP model. Our results also show that, for the whole retina, the changes in local blood supply are only partially responsible for RGC loss following AHIP (~50%), although the degree of correlation increases for peripheral retina. Certainly other mechanisms—as revealed by previous studies, including neurotrophic factor deprivation, glial cell activation, glutamate excitotoxicity, and abnormal immune response—cannot be totally excluded as being responsible for RGC loss following AHIP. It is conceivable that one or several of these mechanisms contribute to the selective loss of RGCs following AHIP.

In conclusion, changes in local blood supply to the rat retina vary from central to peripheral retina following AHIP. Such unequal changes in retinal blood supply might represent at least one cause of selective loss of RGCs.


[22] Nakazawa T, Takahashi H, Shimura M. Estrogen has a neuroprotective effect on axotomized RGCs through ERK signal transduction pathway. *Brain Res.* 2006;1093:141–149.


