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Three experimental glaucoma models in rats: Comparison of the effects of intraocular pressure elevation on retinal ganglion cell size and death

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Abstract

Glaucoma is a chronic and progressive optic nerve neuropathy involving the death of retinal ganglion cells (RGCs). Elevated intraocular pressure (IOP) is considered to be the major risk factor associated with the development of this neuropathy. The objective of the present study was to compare the effects on RGC survival of three different experimental methods to induce chronic elevation of IOP in rats. These methods were: (i) injections of latex microspheres into the eye anterior chamber; (ii) injections into the anterior chamber of a mixture of microspheres plus hydroxypropylmethylcellulose (HPM) and (iii) cauterization of three episcleral veins. The IOP of right (control) and left (glaucomatous) eyes was measured with an applanation tonometer in awake animals. Thirteen to 30 weeks later, RGCs were retrogradely labeled with 3% fluorogold. Subsequently, we analyzed the density of RGCs, as well as the major axis length and area of RGC soma resulting from the application of each method. A significant increase in IOP was found following application of each of the three methods. Cell death was evident in the glaucomatous eyes as compared to controls. However, no statistical differences were found between the extent of cell death associated with each of the three methods. IOP increase also induced a significant increase in the size of the soma of the remaining RGCs. In conclusion, the three methods used to increase IOP induce a similar degree of RGC death. Moreover, the extent of cell death was similar when the retinas were maintained under conditions of elevated IOP for 24 weeks in comparison to 13 weeks.

Keywords: glaucoma; rat retina; retinal ganglion cells; intraocular pressure; episcleral vein; cell death; cell size; microspheres; hydroxipropylmethylcellulose (HPM); Trabecula meshwork; neuronal cell death

1. Introduction

Elevated intraocular pressure (IOP) is one of the most important risk factors for developing glaucoma. In order to understand in detail the mechanisms which take place in glaucoma and lead to retinal damage, fast, inexpensive and reproducible animal models need to be developed (Chew, 1996).

The currently available models of experimental glaucoma involve the induction of a chronic increase in IOP. This increase can be achieved by reducing aqueous humor outflow through the eye. Thus, aqueous humor drainage can be interrupted by cauterizing two or three episcleral veins (Shareef et al., 1995) or by injecting hypertonic saline into the episcleral veins in rats (Morrison et al., 1997). Moreover, laser energy has been employed as a tool to perform burns directed at the trabecular meshwork (TM) (Ueda et al., 1998) and at both TM and episcleral veins (Levkovitch-Verbin et al., 2002). Other methods are based on the blockage of aqueous humor drainage at the level of the trabecular meshwork, avoiding manipulation of the eye vascular system. Thus, injection of different substances such as ghost red blood cells (Quigley and Addicks, 1980) or latex microspheres into the eye anterior chamber (Weber and Zelenak, 2001) leads to TM channel blockade. Finally, injection of viscoelastic agents into the eye anterior chamber has been reported to induce IOP spikes

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by a mechanical obstruction of the trabecular meshwork in rabbits (Benson et al., 1983; Manni et al., 1996; Törngren et al., 2000).

It has been widely demonstrated that retinal ganglion cells (RGCs) are selectively affected during glaucoma. However, few works have analyzed the pattern of RGC death by means of quantification of specifically labeled RGCs (Ko et al., 2001; Morgan et al., 2000; Naskar et al., 2002; Shareef et al., 1995). In this sense, during the first 10 weeks after IOP increase in rats, the rate of RGC death has been estimated to be uniform and almost linear at about 3–4% per week, but the degree of cell death seems to depend on the retinal region analyzed (Laquis et al., 1998).

Apart from RGC death, changes in RGC soma size have been reported in experimental glaucoma. Thus, an overall hypertrophy of all RGC types has been reported in rats after IOP elevation by episcleral vein cauterization (Ahmed et al., 2001). This increase in RGC soma size is also observed after optic nerve crush and axotomy (Moore and Thanos, 1996; Rousseau and Sabel, 2001). The increase in RGC soma size is likely to be in part a response to the space made available by the culling of RGC (Ahmed et al., 2001).

Glaucoma is a chronic and progressive disease and consequently, it is vital to develop experimental models which analyze RGC death following different periods of sustained elevation of IOP. The effect of experimental IOP elevation on RGC death has been analyzed during periods no longer than 12–16 weeks, due to the transitory effect of surgery. The main aim of the present study was to compare the effect of three different glaucoma models on RGC death and soma size. Moreover, we have also compared the effect of different periods of elevation of IOP in the same experimental glaucoma model to determine if a correlation exists between the duration of IOP elevation and the extent of RGC death.

2. Materials and methods

2.1. Animals

Twenty-one female Sprague–Dawley rats weighing 250–300 g were used in the present work. Animals were housed in a standard animal room with food and water provided ad libitum, a constant temperature of 21 °C and a 12-h light/dark cycle. All animal experimentation adhered to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Experiments were designed and carried out in such a way as to minimize animal suffering in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC). Our animal care unit registered in the Animal Users Establishment Register for research in the Basque Country (EU-02-BI). The number of animals was kept to a minimum which was sufficient to generate statistically significant results.

2.2. Control animals

Left eyes of three rats were injected weekly with 20 µl of sterile aqueous solution (0.15 M NaCl; 0.02% Tween 20) into the anterior chamber during 9 weeks.

2.3. Chronic experimental glaucoma model

Glaucoma was induced in the left eye of each animal, whereas the right eye was considered as a control eye. Before surgery, animals were anaesthetized with an intraperitoneal injection of xylazine (Rompún; Bayer, SA, Barcelona, Spain) and ketamine hydrochloride (Ketolar; Parke-Davis, SL, Barcelona, Spain) (7.4 mg/ml and 31.5 mg/ml, respectively). Two drops of 5% povidone yodade were topically applied to the eye before surgery to prevent potential infection. Animals were distributed in three different groups according to the surgical procedure in order to increase IOP:

2.4. Latex microsphere injection

IOP was elevated in the left eye of six rats by injecting 20 µl of a sterile aqueous solution (0.15 M NaCl; 0.02% Tween 20) containing $2–4 \times 10^5$ latex microspheres (FluoSpheres™; Molecular Probes, Eugene, OR; 10 µm diameter) into the anterior chamber. Injections were made using a sterile 30-gauge needle which was initially oriented tangential to the corneal surface and then redirected posteriorly, so it entered into the anterior chamber parallel to the iris. This maneuver introduced for cataract surgery (Langerman, 1994) allows the creation of a self-sealing corneal tunnel and avoids the continuous leaking of beads through the incision. Injections of microspheres were performed weekly until a maintained IOP elevation was observed in the left eye. This elevation was maintained after nine injections in all animals of this group. Animals were kept alive for 30 weeks. Two rats were excluded from this study due to the presence of infectious complications, i.e. corneal abscess and endophthalmitis.

2.5. Injections of latex microspheres with hydroxypropylmethylcellulose (HPM)

Blockade of the trabecular meshwork drainage was achieved in four rats by injecting into the anterior chamber a mixture of microspheres plus HPM. The volume of each injection was approximately 20 µl consisting of 10 µl of 2% HPM (Methocel; Novartis, Annonay, Spain) and 10 µl of microspheres solution prepared as described before, but containing $1–2 \times 10^5$ latex microspheres. Injections were performed weekly until a maintained IOP increase was observed in the left eye. We performed a total of six injections in each rat and animals were kept alive for 30 weeks.

2.6. Episcleral vein cauterization

IOP increase was induced in eight rats by cauterizing three episcleral veins in their left eyes following methods previously described elsewhere (Shareef et al., 1995). Briefly, two dorsal episcleral veins, located near the superior rectus muscle, and one temporal episcleral vein, near the lateral rectus muscle were isolated from the surrounding tissues. A cautery was specifically and precisely applied to the selected vein taking care to avoid thermal damage to the rest of the neighboring tissues.

After each procedure, steroid-antibiotic ointment containing cloramphenicol and dexamethasone (Oftalmolosa cusí De Icol; Alcon Cusí, SA, Barcelona, Spain) was applied to the eye surface. The rats were kept alive for 13 weeks (first group, $N = 4$) and for 24 weeks (second group, $N = 4$).

2.7. IOP measurements in awake animals

The IOP of right and left eyes of awake animals was measured with an applanation tonometer (TonoPen XL, Mentor, Norwell, MA) after the application of tetracaine hydrochloride + oxibuprocaine (Anestesico doble; Coluircusí, Alcon Cusí, Barcelona, Spain). The tonometer was applied perpendicular to the more apical side of the cornea, until at least five or six independent measurements were obtained (each of these values was the average of four readings). The results of the IOP reading were accepted if the confidence interval was greater than or equal to 95%. All measurements were taken at the same hour of the day in order to avoid circadian IOP changes. IOP was analyzed before surgery in order to obtain the baseline values, 1 week after operations and then weekly until the end of the experimental period.

2.8. Fundoscopy

Rats were anesthetized and their pupils were dilated by local application of tropicamide in order to examine the retinal vasculature and verify proper eye perfusion before sacrifice. The cornea was gently applanated with a glass slide to visualize the retina, and the vasculature and optic nerve head was photographed. In this way, the status of the internal circulation of the retina was verified, comparing the vasculature of glaucomatous and control eyes, before sacrificing the animals.

2.9. Retrograde labeling of RGCs

RGCs were retrogradely labeled with 3% fluorogold (Fluorochrome, Inc., Engelwood, CO, USA) diluted in a solution containing 0.9% NaCl and 0.1% dimethylsulfoxide 30 weeks after microspheres or microspheres + HPM injection, and 13 and 24 weeks following episcleral vein cauterization. Twenty microliters of fluorogold solution were injected into the optic nerve of both eyes, 4 mm from optic disc, using a syringe with a 30-gauge needle. Animals were kept alive for 24 h to allow fluorogold to fill the entire population of RGCs. Previous experiments using different time points and different tracers give us 24 h as the best waiting time for fluorogold to trace the complete population of RGCs (Hernandez et al. ARVO 2005). Then, animals were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). Eyes were enucleated and the lens and vitreous were extracted. The eyecups were postfixed with the same fixative for 2 h. After this time retinas were removed and flat-mounted (the retinal ganglion cell layer being uppermost) with PBS/glycerin (1:1) so that shrinkage did not occur during the processing of the tissue.

2.10. Image capture

Images from each retina were obtained using an epifluorescence microscope (Axioskop 2; Zeiss, Jena, Germany) coupled to a digital camera (Coolsnap, RS Photometrics, Tucson, USA). The images were captured in a systematic way, using the optic disc (OD) as reference point. A total of 24 fields was obtained for each retina with the 40× objective lens (0.08 mm^2). The fields were captured in three concentric circle; eight systematically distributed retinal fields were captured.

2.11. Morphometric analysis

The number of RGCs together with the soma major axis length and the soma area of each RGC were analyzed for each recorded field. Analysis of these parameters was performed using a digital palette (Easypen, Genius) in combination with image analysis software (Scion Image; Scion, Frederick, MD). Each RGC soma was filled out directly on the computer screen and values obtained were transferred to a data sheet for subsequent statistical analysis. RGC density was calculated as the number of RGCs in the captured field divided by the size of the field (0.08 mm^2).

Mean values of RGC density and RGC soma area at different retinal regions were calculated. Moreover, we analyzed the distribution of different sized RGCs, defined according to their soma major axis length. The frequency of RGCs with a given major axis length were obtained for each field and retinal region and represented by histograms. RGC density and the size of the soma area were always compared with the control eye from the same rat and in the same retinal region.

Statistical analysis was performed by using the SPSS software (SPSS Sciences, Chicago, IL). IOP, RGC density and soma area were expressed as mean ± SEM. Mean data from glaucomatous retinas were compared with data from control retinas by using Student's t test. ANOVA followed by the Scheffé test was used to compare these parameters in the different retinal regions and different glaucoma models. The minimum level of significant difference was defined as $p < 0.05$.

3. Results

3.1. IOP increase

In control animals, the mean IOP was similar in both the right (23.06 ± 0.8 mmHg) and left eye (23.59 ± 0.7 mmHg) throughout all the experimental period ($p = 0.61$) (Fig. 1A).

Injections of latex microspheres into the eye anterior chamber led to a significant increase in IOP. Thus, the mean IOP in injected eyes was 28.1 ± 0.7 mmHg during the experimental period and this increase reached maximum values during the 27th week when the average IOP during this period was 1.69 times that of the control (37.6 ± 2.6 mmHg treated eye vs. 22 ± 2.5 mmHg in the control eye, $p < 0.05$) (Fig. 1B).

Injections of microspheres + HPM also produced a significant increase in IOP after the 6th week. The mean IOP in

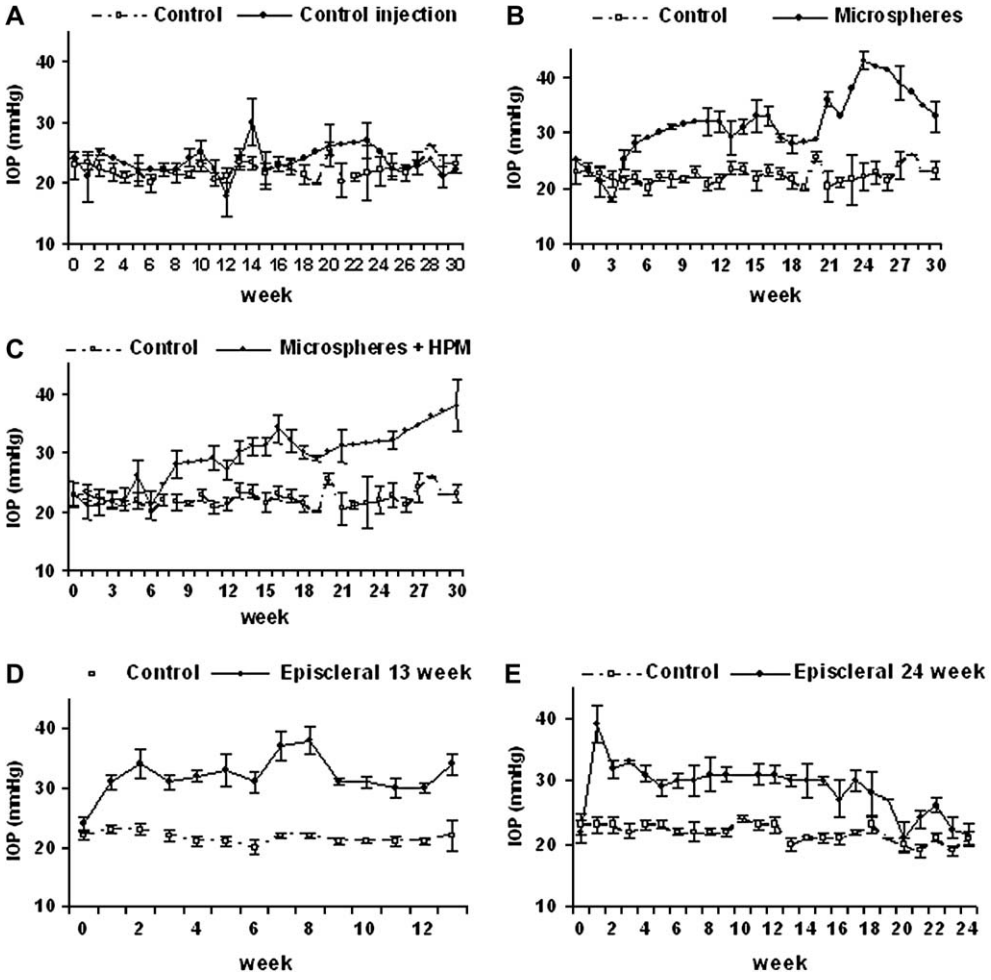

Fig. 1. Changes in intraocular pressure (IOP) following three different methods of experimental glaucoma. Group (A) control animals; the right eye was a control and the left eye was injected with PBS. Group (B) animals were injected weekly with microspheres. Group (C) animals were injected weekly with a mixture of microspheres + HPM. Animals in groups (D) and (E) were subjected to episcleral vein cauterization. All injections or cauterizations were performed on the left (experimental) eye.

injected eyes was 31.1 ± 0.6 mmHg during the experimental period. The largest difference between both eyes was reached by week 27, when the IOP of the injected eye was 1.98 times that of the control eye (42.5 ± 1.7 mmHg vs. 23.0 ± 3.5 mmHg, $p < 0.005$) (Fig. 1C).

Episcleral vein cauterization was the method which led to the fastest increase in IOP. This increase was evident by the first week and remained elevated in the left eyes of these animals until week 17. The largest increase in IOP following cauterization was 1.7 times the control value and this was observed at week 8 (37.5 ± 2.2 mmHg vs. 22 ± 0.6 mmHg, $p < 0.05$) (Fig. 1D). However, a progressive and slow reduction was observed from the 17th week till the end of the experiment. Thus, the mean IOP in treated eyes at this period was more similar to the mean IOP of control eyes (26.7 ± 1.9 mmHg vs. 21.5 ± 0.3 mmHg, $p < 0.05$) (Fig. 1E).

3.2. RGC distribution

RGC density in control eyes varies significantly as a function of the region of the retina being analyzed. Thus, the maximal density of RGCs was observed in the retinal fields closest to the optic nerve where mean RGC density was calculated to be 2624 ± 164 RGC/mm^2. RGC density declines with distance from the optic nerve; thus mean RGC density in the middle retina was 2314 ± 160 RGC/mm^2 ($p < 0.01$ with respect to the central zone). The lowest density of RGCs was found in the most peripheral fields, with a mean RGC density value of 1793 ± 138 RGC/mm^2 ($p < 0.001$ with respect to the central and middle regions) (Table 1, Fig. 2).

Analysis of RGC density in the different experimental groups showed that IOP elevation produces RGC death and that the extent of RGC death induced by the different models of experimental glaucoma is similar, when the whole retina is taken into account. Thus, the extent of death following latex microsphere administration was $23.1 \pm 2.1\%$ of RGCs, while the injection of the microsphere + HPM mixture induced the death of $27.2 \pm 2.1\%$ of the RGC population following 24 weeks of IOP elevation in both treatments. Statistical analysis of these results revealed that there are no significant differences between these treatments ($p = 0.317$). Episcleral vein cauterization induced the death of $27.0 \pm 2.1\%$ of the

Table 1
RGC density and soma size in three different retinal areas in control retinas. RGC density values are expressed as mean (RGCs per mm^2) ± SEM

Retinal area	RGC density (RGC/mm^2)	Soma area (μm^2)
Central retina	2624 ± 164	126.29 ± 1.76
Middle retina	2314 ± 160**	134.28 ± 1.76**
Peripheral retina	1793 ± 38** ##	141.67 ± 1.75** ##
Total average	2234 ± 150	134.08 ± 5.4

RGC soma size values are expressed as μm^2 ± SEM. Statistical differences are represented as: **$p < 0.01$ with respect to the central area; ##$p < 0.01$ with respect to the middle area.

RGCs following 13 weeks of IOP elevation. A similar extent of RGC death was measured following 24 weeks of cauterization (28.5 ± 2.4%, $p = 0.97$) (Table 2).

The extent of RGC death in response to elevated IOP varies according to the zone of the retina analyzed. Thus, the extent of RGC death in the more peripheral regions of the retina was higher than that observed in the more central regions of the papilla. This difference was significant in the episcleral vein cauterization group, in which RGC death in the central zone of the retina was 17.4 ± 2.5%, whereas that in the peripheral regions was significantly higher (33.6 ± 3.6%, $p < 0.01$) (Fig. 2). The injection of microspheres + HPM induced the death of 30.2 ± 3.4% of the RGCs in the peripheral regions of the retina, whereas in the zones proximal to the optic nerve, a smaller extent of death was observed (22.1 ± 2.4%, $p = 0.28$). The death rate following microsphere injection was also higher in the peripheral regions (25.5 ± 5.1%) in comparison to the central regions of the retina (19 ± 2.23%, $p = 0.35$) (Fig. 3).

Analysis of RGC loss following IOP elevation revealed that there were no significant differences between the extent of RGC death in the different quadrants of the retina (dorsal, ventral, nasal y temporal) in any of the different experimental glaucoma models reported herein.

3.3. RGC size

Analysis of the size of the soma of control RGCs showed that in the central zone of the retina, the RGCs are smaller and that the size of the cells increases with eccentricity.

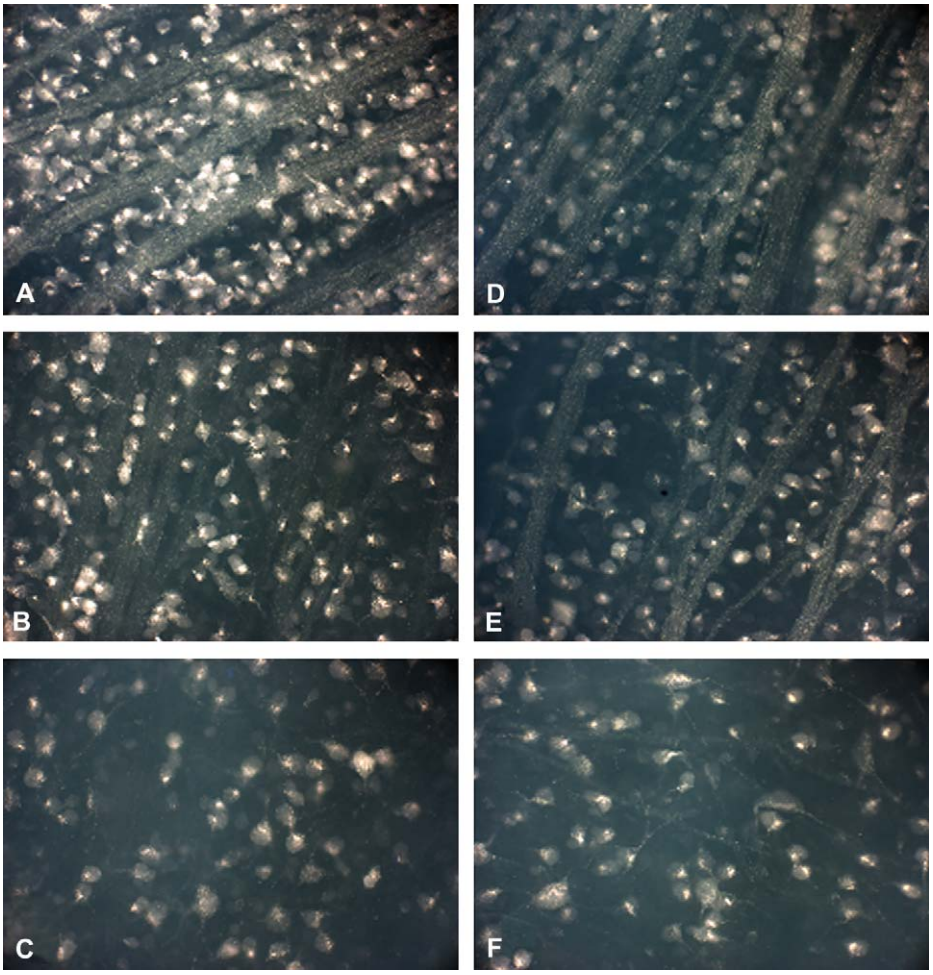

Fig. 2. Fluorogold (3%) labeled retinal ganglion cells in different retinal areas of a control and glaucomatous eye. Notice a gradual decrease in retinal ganglion cell density in peripheral areas in control eyes of central (A), middle (B) and peripheral (C) retinal areas. A similar decrease in RGC density was observed in central (D), middle (E) and peripheral (F) retinal areas subsequent to episcleral vein cauterization. However, reduced cell density was apparent in each of these areas vs. the corresponding areas in control retinas. The scale bar is the same for all figures and represents 50 μm.

Table 2
RGC density in distinct retinal zones in each glaucomatous group

Retinal area	Microspheres		Microspheres + HPM		Episcleral 13 week		Episcleral 24 week	
	Right	Left	Right	Left	Right	Left	Right	Left
Central	3286 ± 107	2696 ± 107**	2562 ± 145	2011 ± 120**	2343 ± 79	2050 ± 64**	2308 ± 83	1708 ± 89**
Middle	2939 ± 119	2484 ± 106*	2499 ± 133	1861 ± 132**	2066 ± 95	1700 ± 88**	1839 ± 86	1431 ± 79**
Peripheral	2244 ± 146	1850 ± 87*	2180 ± 118	1566 ± 101**	1560 ± 86	1183 ± 69**	1284 ± 95	1134 ± 86
Total average	2823 ± 87	2341 ± 71**	2412 ± 78	1819 ± 72**	1990 ± 60	1644 ± 55**	1808 ± 70	1425 ± 56**

RGC density values are expressed as mean (RGCs per mm^2) ± SEM. Results from statistical analysis are represented as: *$p < 0.05$, **$p < 0.01$ significant difference with respect to each control eye.

Thus, the average area of the RGC soma in the central zone was measured to be 126 ± 1.76 μm^2, whereas this value was significantly larger in the middle region of the retina (134.28 ± 1.76 μm^2; $p < 0.01$). The largest sized RGCs were found in the most peripheral retina. In this region, the mean area of the RGC soma was significantly bigger than in the other two regions of the retina ($p < 0.01$) with a value of 141.67 ± 1.75 μm^2 (Table 1).

The increase in IOP induced by injections of microspheres + HPM and by episcleral vein cauterization produced significant changes in the mean size of RGC somas (Fig. 4). Thus, repeated injections of microspheres + HPM produced an increase (11.9 ± 1.46%) in the mean area of the RGC soma, whereas in the cauterized group, the increase in size reached 16 ± 1.8% at week 24. This increase in the mean size of the soma is likely due to a total hypertrophy of distinct RGCs with different axial longitudes (Fig. 5). Significant changes in the mean area of the RGC soma following microsphere injection or in animals 13 weeks after cauterization of the episcleral veins were not detected.

4. Discussion

IOP measurements were made using a tonopen XL applanation tonometer, which permits non-invasive determinations of IOP (Moore et al., 1993). Following the methodology used by others (Benozzi et al., 2002; Cohan and Bohr, 2001; Jia et al., 2000a), IOP measurements were performed using only topical anesthesia, thus avoiding any hypotensive (Jia et al., 2000b) or even neuroprotective effects (Fujikawa, 1995; Ozden and Isenmann, 2004) associated with general anesthesia. IOP determinations were always performed at the same time of the day, with a view to avoiding pressure fluctuations associated with the circadian rhythm and the light/dark cycles to which the rats were subjected (Jia et al., 2000b; Krishna et al., 1995; Moore et al., 1996).

The mean IOP which we measured in control eyes (23.06 ± 0.8 mmHg) was slightly higher than that reported by other authors (15 mmHg) although most of them had used different species of rats (Ahmed et al., 2001; Bayer et al., 2001; Laquis et al., 1998; Woldemussie et al., 2001). Curiously, the mean IOP of glaucomatous eyes reported by other authors is lower than that observed in the present study following IOP elevation using the three different methods. These discrepancies could be due to differences in rat strains or also, more likely to the use of general anesthesia in the cited studies. In any event, IOP elevation measured in the present study following the induction of glaucoma is similar to that reported by other authors (Laquis et al., 1998; Naskar et al., 2002; Woldemussie et al., 2001) (Table 3).

The present results indicate that in animal groups subjected to the injection of microspheres alone and microspheres together

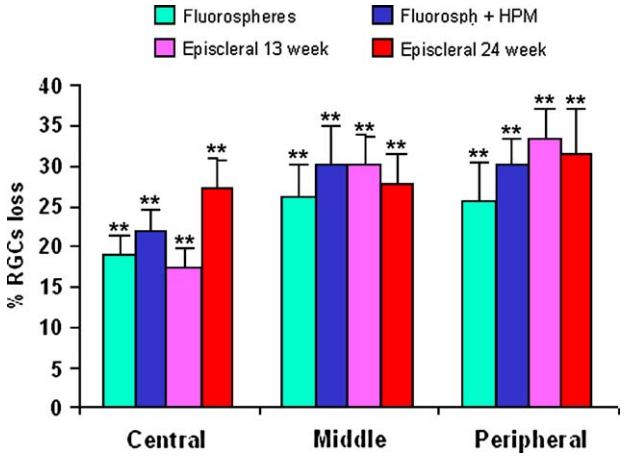

Fig. 3. Percentage of RGC loss in three different retinal areas for the four types of experimental glaucoma. Results from statistical analysis are represented as: *$p < 0.05$, **$p < 0.01$ significant difference with respect to each control eye (right eye).

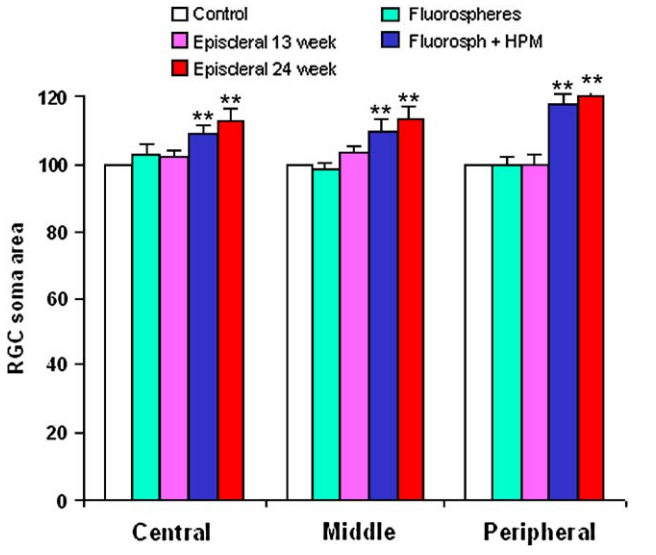

Fig. 4. Comparison of RGC soma area in response to IOP elevation. Results from statistical analysis are represented as: *$p < 0.05$, **$p < 0.01$ significant difference with respect to each control (100).

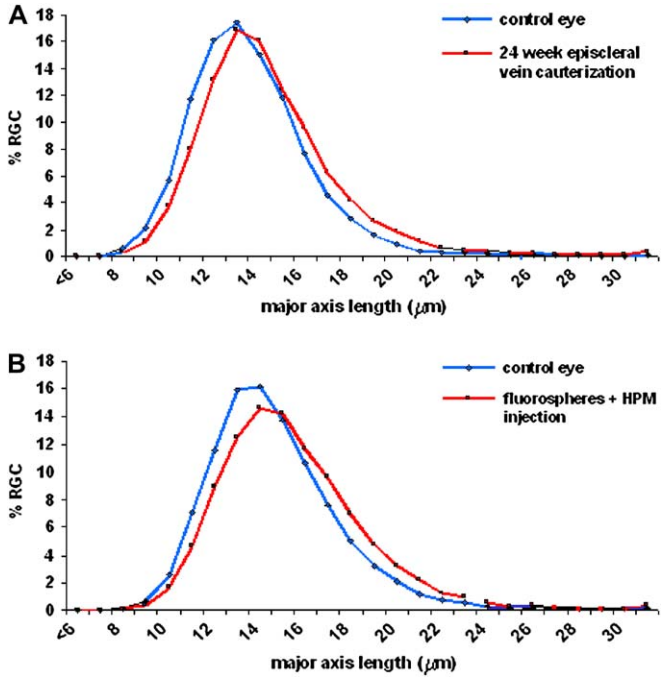

Fig. 5. Comparison of RGC mayor axis length in response to different types of IOP elevation. Group (A) episcleral vein cauterization and (B) microspheres + HPM injection group. Sizes of all RGCs are compared with respect to each control retina.

with HPM, nine and six injections respectively are necessary to achieve a sustained increase in IOP. The number of microsphere injections necessary to induce a sustained elevation in rat IOP is similar to that which has been reported for the monkey (Weber and Zelenak, 2001). Regarding episcleral vein cauterization, we observed IOP elevation during the first week, with IOP values similar to those obtained using the two former methods. However, IOP induced with this method progressively drops from the 17th week onwards and by week 24, the IOP is similar to that of the control eye. In contrast, this IOP normalization is not observed in the two groups which involve injections into the anterior chamber; in these groups, elevated IOP was observed throughout the course of the experiment. In this regard, other authors have reported a normalization of IOP 8 weeks after cauterization of the episcleral and vorticose veins (Grozdanic et al., 2003). This reduction in IOP has also been reported in other models of experimental glaucoma, such as photocoagulation of the trabecular meshwork using laser (Levkovitch-Verbin et al., 2002; Ueda et al., 1998), and even in a glaucoma model in the mouse DAB/2NNia strain (Danias et al., 2003). It has been suggested that the application of 5-fluoruracil following episcleral vein cauterization helps to maintain elevated IOP for longer periods (Mittag et al., 2000).

Our results indicate that there is large variability in the density of RGCs between the retinas of different rats, but not between the two retinas of the same rat. This finding corroborates that of other authors who have used the pig (García et al., 2005; Ruiz-Ederra et al., 2005) or rat (Danias et al., 2002) as reference models. Analysis of the pattern of distribution of RGCs in the control retina reveals that the distribution of RGCs in the rat is not homogeneous, but rather that there is a progressive reduction in the density of cells towards more peripheral areas. Consequently, when comparing RGC density, it is necessary to compare retinal fields in the two retinas of the same rat. Despite the fact that we observed certain differences in the proportion of RGC death for each of the studied groups ($23.1 \pm 2.1\%$ in the microsphere injection group; $27.2 \pm 2.1\%$ in the microsphere + HPM group and $28.5 \pm 2.4\%$ in the episcleral vein cauterization group), these differences between the three glaucoma models are not

Table 3
Summary of the studies carried out to date using the rat glaucoma model

Author	Glaucoma model	Rat strain	IOP control	IOP glaucoma	General anesthesia	RGC label
Laquis et al., 1998	Episcleral vein cauterization	Wistar	13 mmHg	21–23 mmHg	Mildly anesthetized	Fast blue
Jia et al., 2000a	Hypertonic saline injection	Brown-Norway	Light phase 21 mmHg, dark phase 31 mmHg	Light phase 22 mmHg, dark phase 39 mmHg	Awake	None
Jia et al., 2000b	Hypertonic saline injection	Brown-Norway	Awake 20 mmHg		50% reduction in IOP after 5 min ketamine injection	None
Bayer et al., 2001	Episcleral vein cauterization	Wistar	13 mmHg	36 mmHg	Mixture (AXK) Acepromazine + xylacine + ketamine injection	Fluorogold
Woldemussie et al., 2001	Trabecular meshwork laser photo-coagulation	Wistar	16 mmHg	32–35 mmHg	Acepromazine injection	Dextranamine
Ahmed et al., 2001	Episcleral vein cauterization	Wistar	12–14 mmHg	28–30 mmHg	Mixture (AXK) Acepromazine + xylacine + ketamine injection	Fluorogold
Levkovitch-Verbin et al., 2002	Trabecular meshwork laser photocoagulation	Wistar	19 mmHg	25 mmHg	Ketamine + xylacine injection	None
Naskar et al., 2002	Episcleral vein cauterization	Sprague-Dawley	15 mmHg	25 mmHg	Ether inhalation	4-Di-10ASP
Benozzi et al., 2002	Hyaluronic acid intracameral injection	Wistar	11 mmHg	21 mmHg	Awake	None
Grozdanic et al., 2003	Episcleral and vortex vein cauterization	Brown-Norway	25 mmHg	34 mmHg	3% halothane + 30% NO + 70% O_2	None

The authors, the rat strain used, the model of glaucoma, IOP data, type of anesthesia used and tracer to label the RGCs are specified.

statistically significant. Regarding RGC death in the different zones of the retina, we observed that the rate of cell death is higher in the peripheral areas and varies from $33.6 \pm 3.6\%$ in the vein cauterized group to $25.5 \pm 5.1\%$ in the microsphere injected group. These values are similar to those reported following 8 and 10 weeks of vein cauterization (Ahmed et al., 2001).

On the other hand, the degree of RGC death following episcleral vein cauterization is similar on weeks 13 and 24 ($27.0 \pm 2.1\%$ vs. $28.5 \pm 2.4\%$ respectively). This finding may be due to the normalization of the IOP from the 17th week onwards. A similar finding has also been reported with the DBA/2 mouse strain in which, following various months of elevated IOP and the progressive loss of RGCs, a normalization of IOP value and a reduced degree of cell death were observed (Danias et al., 2003).

We found that the size of RGCs increases significantly by 24 weeks following episcleral vein cauterization or microsphere + HPM injection. In these groups, the degree of RGC death is also somewhat higher than the rest and the increase in cell size occurs essentially in regions in which cell death is more extensive. Indeed, a direct correlation has been reported between the reduction in RGC density and the increase in cell soma area (Manni et al., 1996). Increased RGC soma area has been reported following episcleral vein cauterization; a global hypertrophy of RGCs was observed over 4–10 weeks (Ahmed et al., 2001). Similarly, increased RGC soma area has been reported following complete axotomy of the optic nerve and as a compensatory reaction following optic neuropathy due to nerve crush (Moore and Thanos, 1996; Rousseau and Sabel, 2001). It has been postulated that the increase in the area of the soma may be related to the progressive loss of RGCs and to an altered cellular behavior of RGCs in response to a distinct tissue environment (Moore and Thanos, 1996; Rousseau and Sabel, 2001). Moreover, it is likely that surviving RGCs in elevated IOP retinas were not degenerating cells since their branching field diameter enlarged in some cases after damage (Ahmed et al., 2001).

Analysis of histograms in Fig. 4 reveals that the hypertrophy of RGC somata is global, occurring in cells in each region of the retina. However, in the groups involving intraocular injection, especially in that which involves HPM which induces pressure peaks, a larger degree of hypertrophy appears to occur.

It should be remembered that the hypertensive effect of substances which are injected weekly to progressively obstruct the trabecular meshwork may also be due in part to inflammatory trabeculitis (Weber and Zelenak, 2001). It is of course vital also to perform careful surgery during the separation of the extraocular muscles, in order to avoid severing the anterior ciliary veins which could result in ischemia of the anterior chamber (Ino-ue et al., 1999; Koeugh et al., 1983; Korzycka et al., 2000; Ben Simon and Abraham, 2004).

In conclusion, IOP elevation and cell death observed following episcleral vein cauterization are similar to those observed using the other two methods reported herein. However, the risk of complications is reduced with the cauterization method. On the basis of the present study, we can make the following recommendations: injection-based methods are more appropriate to study chronic IOP effects, whereas episcleral vein cauterization (EVC) could be a very useful model for the study of neuroprotective trophic factor cocktails, since IOP normalizes without any further interventions by week 24; only one intervention is required and elevated IOP occurs rapidly. Thus one could compare cell death following IOP elevation due to EVC in the presence and absence of cocktails.

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