

Optic neuropathy and increased retinal glial fibrillary acidic protein due to microbead-induced ocular hypertension in the rabbit

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Received: 2016-05-13 Accepted: 2016-09-19

Abstract

• **AIM:** To characterize whether a glaucoma model with chronic elevation of the intraocular pressure (IOP) was able to be induced by anterior chamber injection of microbeads in rabbits.

• **METHODS:** In order to screen the optimal dose of microbead injection, IOP was measured every 3d for 4wk using handheld applanation tonometer after a single intracameral injection of 10 μ L, 25 μ L, 50 μ L or 100 μ L microbeads (5×10^6 beads/mL; $n=6$ /group) in New Zealand White rabbits. To prolong IOP elevation, two intracameral injections of 50 μ L microbeads or phosphate buffer saline (PBS) were made respectively at days 0 and 21 ($n=24$ /group). The fellow eye was not treated. At 5wk after the second injection of microbeads or PBS, bright-field microscopy and transmission electron microscopy (TEM) were used to assess the changes in the retina. The expression of glial fibrillary acidic protein (GFAP) in the retina was evaluated by immunofluorescence, quantitative real-time polymerase chain reaction and Western blot at 5wk after the second injection of microbeads.

• **RESULTS:** Following a single intracameral injection of 10 μ L, 25 μ L, 50 μ L or 100 μ L microbead, IOP levels

showed a gradual increase and a later decrease over a 4wk period after a single injection of microbead into the anterior chamber of rabbits. A peak IOP was observed at day 15 after injection. No significant difference in peak value of IOP was found between 10 μ L and 25 μ L groups (17.13 ± 1.25 mm Hg vs 17.63 ± 0.74 mm Hg; $P=0.346$). The peak value of IOP from 50 μ L group (23.25 ± 1.16 mm Hg) was significantly higher than 10 μ L and 25 μ L groups (all $P<0.05$). Administration of 100 μ L microbead solution (23.00 ± 0.93 mm Hg) did not lead to a significant increase in IOP compared to the 50 μ L group ($P=0.64$). A prolonged elevated IOP duration up to 8wk was achieved by administering two injections of 50 μ L microbeads (20.48 ± 1.21 mm Hg vs 13.60 ± 0.90 mm Hg in PBS-injected group; $P<0.05$). The bright-field and TEM were used to assess the changes of retinal ganglion cells (RGCs). Compared with PBS-injected group, the extended IOP elevation was associated with the degeneration of optic nerve, the reduction of RGC axons (47.16%, $P<0.05$) and the increased GFAP expression in the retina (4.74 ± 1.10 vs 1.00 ± 0.46 , $P<0.05$).

• **CONCLUSION:** Two injections of microbeads into the ocular anterior chamber of rabbits lead to a prolonged IOP elevation which results in structural abnormality as well as loss in RGCs and their axons without observable ocular structural damage or inflammatory response. We have therefore established a novel and practical model of experimental glaucoma in rabbits.

• **KEYWORDS:** microbead; ocular hypertension; optic neuropathy; glial fibrillary acidic protein; rabbit

DOI:10.18240/ijo.2016.12.05

Zhao J, Zhu TH, Chen WC, Peng SM, Huang XS, Cho KS, Chen DF, Liu GS. Optic neuropathy and increased retinal glial fibrillary acidic protein due to microbead-induced ocular hypertension in the rabbit. *Int J Ophthalmol* 2016;9(12):1732-1739

INTRODUCTION

Glaucoma is a relatively common neurodegenerative disease, estimated to affect over 80-million people worldwide^[1-2]. The disease is characterized by optic nerve damage and retinal ganglion cell (RGC) degeneration

leading to visual field defect or even blindness^[3-4]. This study aims at constructing a reliable glaucoma model in rabbits in order to further clarify molecular processes leading to the development of glaucoma as well as a suitable model for the pharmacokinetic or pharmacodynamics study.

Mice, rats, rabbits and monkeys are the animals most commonly used to construct artificial models of glaucoma^[5-8]. The bigger size of rabbit eyeball poses significant convenience to operate on and manipulate while rabbit care remains relatively cost-effective^[9]. The leporine glaucoma model has been receiving dramatic attention in past few years^[10]. Momentarily two validated methods for inducing glaucoma in rabbits are being employed. The first one causes intraocular pressure (IOP) elevation by intracameral injection with carbomer^[11], subconjunctival injection of betamethasone^[12-13] and posterior chamber injection of α -chymotrypsin^[14-15]. Another technique to obstruct humor outflow is the destruction of the angular meshwork with a laser^[16-18]. However, both methods result in severe inflammatory responses in the anterior segment, and will result in many complications. Thus, a safe, reliable, and cost-effective method to induce elevated IOP is yet to be developed.

Previous studies have shown that IOP elevation can be induced by injection of fluorescent polystyrene microbeads into the ocular anterior chamber in mice and rats^[19-23]. Therefore we sought to investigate whether fluorescent polystyrene microbead could be used to induce chronic IOP elevation in rabbits for the establishment of a reliable leporine model of glaucoma. Furthermore, the glial fibrillary acidic protein (GFAP), a marker for astrocytes and Müller cell activation^[24], was used for monitoring the progression of glaucomatous neurodegeneration in the rabbit model with ocular hypertension (OHT).

MATERIALS AND METHODS

All experimental procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal ethics approval was obtained from the Animal Ethics Committee at the Shenzhen Eye Hospital Affiliated to Jinan University.

Animal Use Male New Zealand White rabbits (aged 8 to 12wk and weighed 1.8 to 2.0 kg) were purchased from Guangdong Province Experimental Animal Center (China). All rabbits were housed in a 12h light (50 lux illumination) and 12h dark (<10 lux illumination) cycle (light was turned on at 7 a.m.), with *ad libitum* access to food and water. No rabbit became ill prior to the experimental endpoint. The health and well-being of the rabbits were monitored. Minor stress induced by operation was looked for daily by observing the motility, grooming and feeding behavior after injections. Typical behaviors that indicate discomfort include agitation and excessive rubbing of the eye^[25].

A Single Injection of Microbeads and Intraocular Pressure Measurement for Dose Screening In order to assess the optimal amount of microbeads to induce stable IOP elevation, a total of 24 New Zealand White rabbits were randomized into 4 groups and received a single intracameral injection of 10 μ L, 25 μ L, 50 μ L or 100 μ L microbeads (a diameter of 15 μ m; FluoSpheres; Invitrogen, Carlsbad, CA, USA) reformulated in phosphate buffered saline (PBS) at a concentration of 5×10^6 beads/mL. Rabbits were anesthetized with an intramuscular injection of xylazine hydrochloride (4 mg/kg, DunhuaKuta Animal Pharmaceutical Co., Ltd., China) supplemented with a topical anesthetic agent on the operated eye (0.5% cocaine hydrochloride ophthalmic solution, Zhongshan Ophthalmic Centre, Sun Yat-Sen University, China). The corneal limbus was gently punctured using a 30-gauge needle to generate easy tunnel entry for microsyringe injection. A certain amount of aqueous humor (150 μ L) was discharged to avoid the acute IOP spike immediately after injection, and an air bubble (50 μ L) was subsequently injected for sustaining the anterior chamber via the entry. A various volume (10-100 μ L) of microbead solution was injected through this preformed tunnel hole into the anterior chamber by a Hamilton syringe. Antibiotic drops (0.3% ofloxacin ophthalmic solution; Hangzhou Minsheng Pharmaceutical Co., Ltd., China) were placed on the operated eye to prevent infection after injection. No treatment was administered to the left eye in order to provide an internal control.

The IOP measurements were performed and recorded in conscious rabbits with the topical anesthesia placed on each eye (0.5% cocaine hydrochloride ophthalmic solution). IOP was measured every 3d for 4wk on a total of 10 occasions after injection. A handheld applanation tonometer (Accutome Inc., Malvern, PA, USA) was used to measure IOP. The average of three consecutive measurements was used as final IOP value. All IOP measurements were performed at a similar time during the day (7 to 9 p.m.) to exclude circadian variations.

Two Injections of Microbeads to Induce a Prolonged Intraocular Pressure Elevation After examining the screening data, it was decided to use 50 μ L microbead (5×10^6 beads/mL) to induce an IOP elevation. A total of 48 rabbits were randomly assigned to receive two intracameral injections of either 50 μ L of microbeads or PBS in the right eye as OHT or control group at days 0 and 21, while no treatment in the left eye was administered. The IOP was measured at day 1 and following every 3d for 8wk using a handheld applanation tonometer as described in the single injection experiment.

The morphology of anterior segment of the eye was checked by a slit-lamp equipped camera (SLM-8; Kanghua Science & Technology Co., Ltd., China) every 3d throughout the

experiment and corneal endothelial microscopy (SP-3000P; TOPCON, Japan) at 1wk after the first injection.

Anterior Chamber Angle and Retinal Observations with Fluorescence Microscope The rabbits were sacrificed at 5wk after the second injection. The tissues of chamber angle and the retina were obtained and embedded in optimal cutting temperature compound (OCT; Shanghai solarbio Bioscience & Technology Co., Ltd., China) for further sectioning and staining. Ten μm frozen sections were fixed with 4% paraformaldehyde (Beijing solarbio science & technology Co., Ltd., China) at room temperature for 15min and then subjected to the counterstained with DAPI. The sections were observed and photographed under a fluorescence microscope (AXIO Vert.A1; Carl Zeiss).

Retinal Ganglion Cell and Optic Nerve Visualization Five weeks after the second injection, the retina and optic nerve were obtained immediately and fixed in 4% paraformaldehyde for 24h. The optic nerve was excised 1.5 mm posterior to the eyeball and cross sectional slices were constructed. The retina was excised at the 6 and 12 o'clock sectors relative to the optic disc. The prepared optic nerve and retina segments were then embedded in paraffin and sectioned (10 μm). Sections were stained with hematoxylin and eosin (H&E) and photographed with a microscope system (N-STORM; Nikon, Japan) at 40 \times magnification.

Transmission Electron Microscope Assessment of Optic Nerve Sections The optic nerve were isolated immediately after rabbit sacrifice and fixed in 2.5% glutaraldehyde phosphate buffer (Beijing solarbio science & technology Co., Ltd., China) for 2h. With light microscopic localization, ultrathin 100 nm sections were constructed from the 1 μm section. The ultrathin sections were stained with uranyl acetate and lead citrate, and scanned with transmission electron microscope (TEM; JEM-100S; FEI, USA). At 5800 \times magnification twelve rectangular images measuring 246 μm^2 each were randomly selected from each optic nerve section. Axonal densities were calculated by averaging the sum of the number of axons from each of the TEM images and dividing that number by the area. The ultrastructure of optic nerve was visualized with the TEM at 40 000 \times magnification. Images were processed with Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc. Silver Spring, MD, USA) [26]. The loss of percentual axons was calculated by dividing the number of axons in the optic nerve of microbead-injected eye by the PBS-injected eye.

Identification of Astrocytes and Müller Cell Activation by Glial Fibrillary Acidic Protein For the purpose of immunofluorescence staining, the retina was embedded in OCT and sectioned into 10 μm by using a freezing microtome (Leica CM1860, Germany). The retinal tissue was incubated with a primary antibody against a GFAP-specific marker (1:1000 dilution, Sigma-Aldrich, USA),

followed by an Alexa Fluor 488 conjugated secondary antibody (Invitrogen, USA). Expression of GFAP was examined under a fluorescent microscope (AXIO Vert.A1; Carl Zeiss).

TRIZOL reagent (Invitrogen) was used to extract retinal mRNA for quantitative real-time polymerase chain reaction (qPCR) analysis. One μg mRNA was reversely transcribed into cDNA and thereafter amplified by qPCR. Relative expression of GFAP mRNA was analyzed using the $2^{-\Delta\Delta\text{CT}}$ method [27]. β -actin gene served as an internal reference to exclude sampling error. The sequences of the primers are as follows: GFAP, forward 5'-ACCTGCTCAACGTGAAGCTG-3', reverse 5'-GGTCTTACCACGATGTTCC-3'; β -actin, forward 5'-ACGACATGGAGAAGATCTG-3', reverse 5'-TGTGAACGTCTCGAACATG-3'.

Retinal protein was extracted for the quantification of intracellular GFAP by Western blot analysis [28]. Protein extracts were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel and incubated with primary anti-GFAP (1:1000 dilution, Sigma-Aldrich) or anti- β -actin antibodies (1:1000 dilution, Sigma-Aldrich) for 12h at 4 $^{\circ}\text{C}$. Then a goat anti-rabbit secondary antibody-IgG (1:1000 dilution, Invitrogen) was added at room temperature for 1h. Following X-ray exposure, optical density of GFAP and β -actin was measured (Bio-Rad, ChemiDoc MP System, CA, USA).

Statistical Analysis Data are expressed as mean \pm standard deviation. Mean data were analyzed with unpaired t -tests or two-way analysis of variance (ANOVA) followed by post-hoc Tukey analysis (SPSS software, version 18.0, SPSS Inc., Chicago, IL, USA). A P value <0.05 was considered statistically significant.

RESULTS

Distribution of Microbeads After an Intracameral Injection Scattering microbeads (orange) were observed in the anterior chamber immediately after a single intracameral injection of 50 μL microbeads (Figure 1A). No overt corneal or iris injury was found after injection and no opaque cornea, edema or exudation in the anterior chamber throughout the experiments was found in the rabbits (Data not shown). Corneal endothelial microscopy showed the normal features of corneal endothelium with regular hexagon cell morphology and clear boundaries at 1wk after injection of 50 μL microbeads (Figure 1B). The accumulation of microbeads was observed in the anterior chamber angle at 8wk after injection (Figure 1C, 1D).

Elevation of Intraocular Pressure Following a Single Injection of Microbeads IOP levels showed a gradual increase and a later decrease over a 4wk period after a single injection of microbead (10 μL , 25 μL , 50 μL or 100 μL) into the anterior chamber of rabbits ($n=6/\text{group}$). A peak IOP was observed at day 15 after injection. The peak values of IOP in each group were at day 15 as following: 17.13 \pm 1.25 mm Hg in

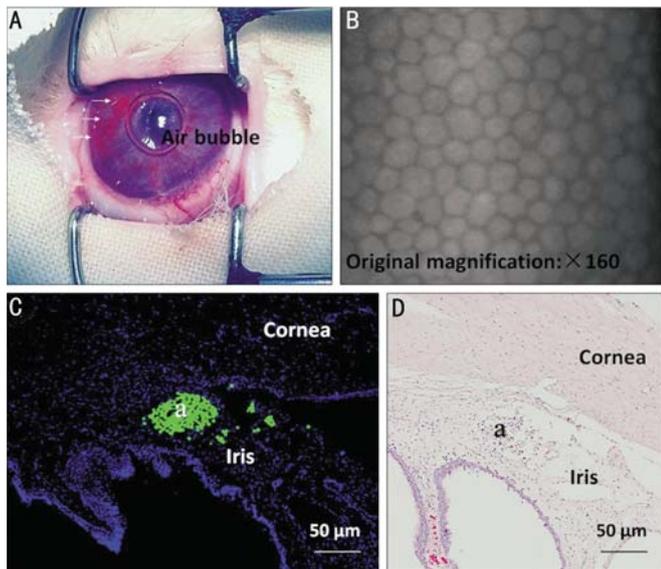


Figure 1 Distribution of microbeads in the anterior chamber following an intracameral injection A: The representative image on scattering microbeads taken immediately after injection of 50 µL microbeads (orange). White arrowheads indicate microbeads; B: The representative image of corneal endothelium was taken at 1wk after injection of 50 µL microbeads; C, D: Accumulation and localization of FITC-labelled microbeads (a) in the anterior chamber angle at 8wk after the first injection. Scale bars: 50 µm.

the 10 µL group, 17.63±0.74 mm Hg in the 25 µL group, 23.25±1.16 mm Hg in the 50 µL group and 23.00±0.93 mm Hg in the 100 µL group (Figure 2). There is no significant difference in peak value of IOP demonstrated between 10 µL and 25 µL groups ($P=0.346$). The peak value of IOP from 50 µL group was significantly higher than the 10 µL and 25 µL groups (all $P<0.05$). Administration of 100 µL microbead solution did not lead to a significant increase in IOP compared with the 50 µL group ($P=0.64$). The IOP levels of all control (Ctrl) eyes (13.60±0.90 mm Hg) remained stable throughout the experiments.

Extension of Intraocular Pressure Following Two Injections of Microbeads

Following the results of the above screening, an amount of 50 µL microbeads was used to research extended IOP elevation. The anterior chamber of the right eye was injected with either 50 µL microbeads or PBS ($n=24$ /group) at days 0 and 21 (Figure 3). A first peak IOP value (23.83±1.14 mm Hg) was reached 15d after the first injection. A second peak (23.26±1.03 mm Hg) appeared at day 30 and then gradual decrease of the IOP to baseline level was observed up to 8wk after the first injection. The IOP did not alter in the PBS-injected (control) group (13.61±0.90 mm Hg) and significantly lower compared with the two-injection group (20.48±1.21 mm Hg, $P<0.05$).

Pathological Changes in the Optic Nerve and Retina Associated with Microbead-induced Ocular Hypertension

The H&E staining optic nerve sections from

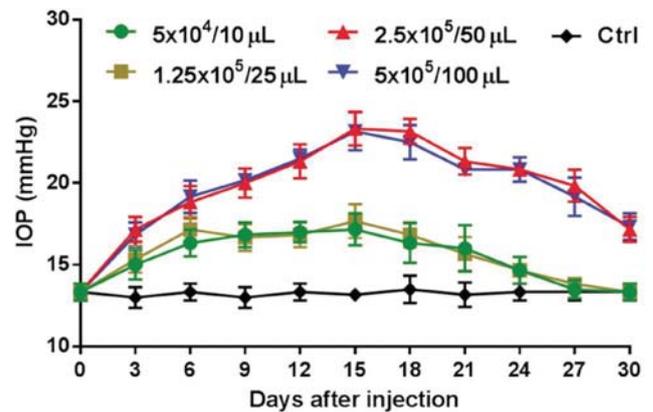


Figure 2 Effect of the IOP levels after the intracameral injection of microbeads The elevation of IOP was assessed after a single injection of microbeads with different volumes (10 µL, 25 µL, 50 µL or 100 µL) into the ocular anterior chamber of rabbit ($n=6$ /group).

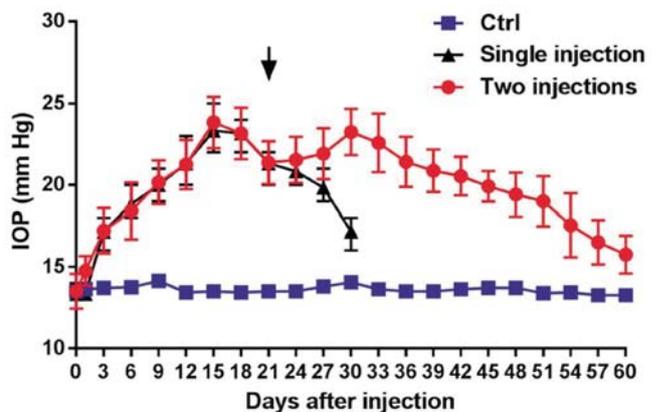


Figure 3 Assessment of IOP elevation following two injections of microbeads The elevation of IOP was assessed after two injections of microbeads (circle) or PBS (square) into the ocular anterior chamber of rabbit ($n=24$ /group). Arrowhead indicates the repeat injection. The IOP levels after the single injection of 50 µL microbeads (triangle).

OHT (microbead-injected) group showed an apparent decrease on the density of nerve fibers, and the TEM images exhibited signs of degeneration with axon swelling, disruption of axon fascicles, increases in connective tissue septa and lower axon numbers (Figure 4A). There was a statistically significant difference in the axon density between the control group ($52.65 \pm 4.83 \times 10^4$ axons/mm²) and OHT group ($27.82 \pm 2.54 \times 10^4$ axons/mm²) ($P<0.05$; Figure 4B). The extent of axonal loss was determined to be 47.16%±2.35% in the OHT group compared to control group. There was no statistically significant difference in the cross-sectional area of optic nerve between control group (0.68 mm²) and OHT group (0.67 mm²) ($P=0.72$; Figure 4C).

The histology of retinal layers in control eyes appeared to be normal and exhibited relatively dense and orderly RGCs in ganglion cell layer. In contrast, sparse RGCs were observed in the microbead-injected eyes (Figure 5).

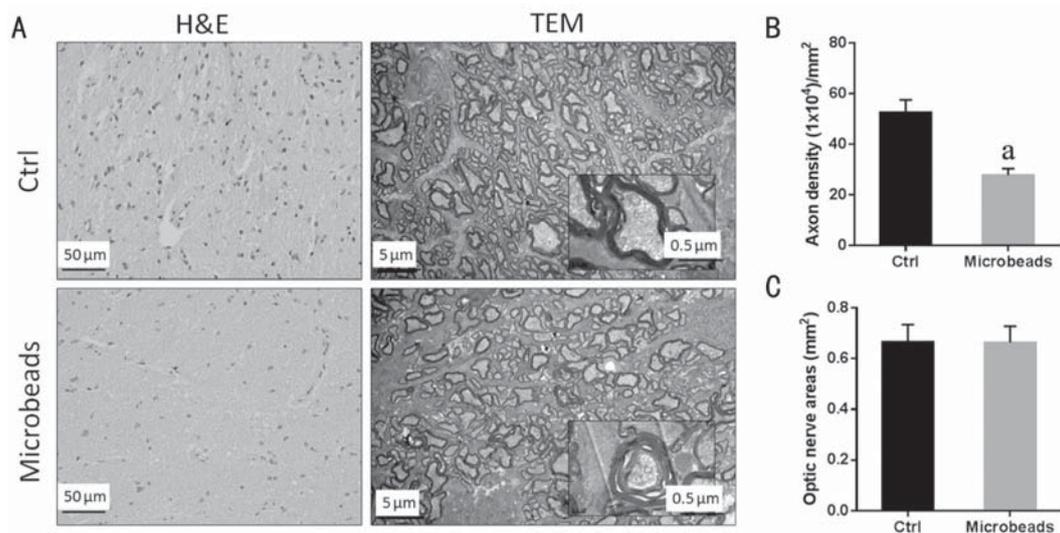


Figure 4 Optic nerve abnormality associated with microbead –induced OHT A: The H&E staining and the TEM images of cross-sections through the optic nerve after two intracameral injections of PBS or microbeads; B: Mean axon density; C: Cross-sectional area of optic nerve from rabbits receiving two intracameral injections of PBS or microbeads ($n=6/\text{group}$; ^a $P<0.05$ vs PBS-injected group).

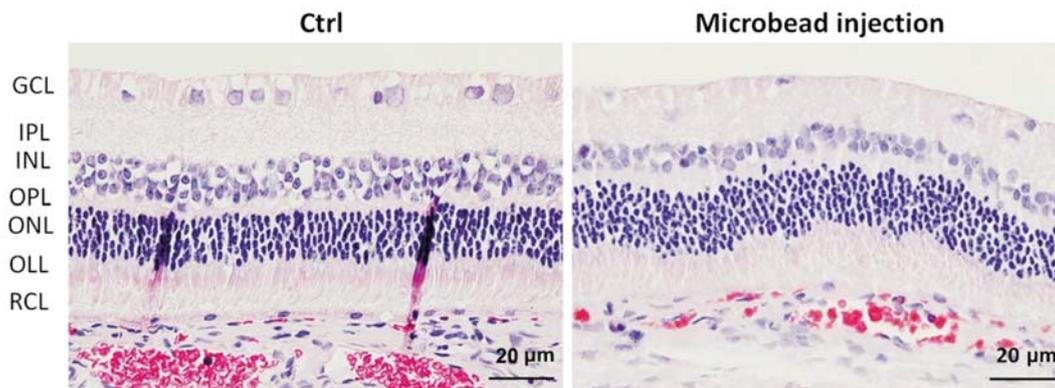


Figure 5 Retinal degeneration associated with microbead –induced OHT The cross-sections of retina obtained from the rabbits receiving two intracameral injections of PBS or microbeads with H&E staining. GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; OLL: Outer limiting membrane layer; RCL: Rods and cones layer. Scale bars: 20 μm .

Upregulation of Glial Fibrillary Acidic Protein Associated with Microbead –induced Ocular Hypertension Immunofluorescent staining of retina showed a background GFAP expression on astrocytes in the control eyes. In contrast, a greater proportion of microbead-injected eyes showed strong activation, which is evident GFAP immunoreactivity on astrocytes and Müller cell processes, spanning throughout the whole retinal layers (Figure 6A). The mRNA and protein expression of GFAP were detected by qPCR and Western blot respectively. Both protein (Figure 6B; $P<0.05$) and mRNA (Figure 6C; Table 1; $P<0.05$) of GFAP were significantly higher in the OHT group compared to control group.

DISCUSSION

The aim of this study was to develop a rabbit model of open angle glaucoma applying an effective and reproducible method modified from recent studies of microbead injection to induce IOP elevation in rodents [19-21]. A single injection of 50 μL microbeads to the rabbit anterior chamber induced

IOP elevation up to 4wk, while a second injection of microbeads at day 21 extended the period to 8wk, resulting in RGC degeneration and up to 47% axon loss that mimic glaucomatous changes, without causing obvious ocular structure damage or inflammatory responses. Up-regulation of GFAP was observed in microbead-induced OHT eyes at week 8, which was also found in previous studies such as rodent models of acute [29-30] and chronic IOP elevation [31] as well as in human glaucoma[32].

A previous study verified that a single 2 μL injection of either 10 or 15 μm microbeads into the anterior chamber of mice could successfully induce IOP elevation 50% above baseline in 2d [19]. Since the different sizes of the eyeball in rabbits, it was hypothesized that a larger amount and bigger size of microbeads is needed to induce OHT. We have explored the IOP changes after injection of various volumes (10-100 μL) of 15 μm microbeads into the anterior chamber of rabbits, and have found that 50 μL of microbeads is the optimized volume to induce a significant increase in mean

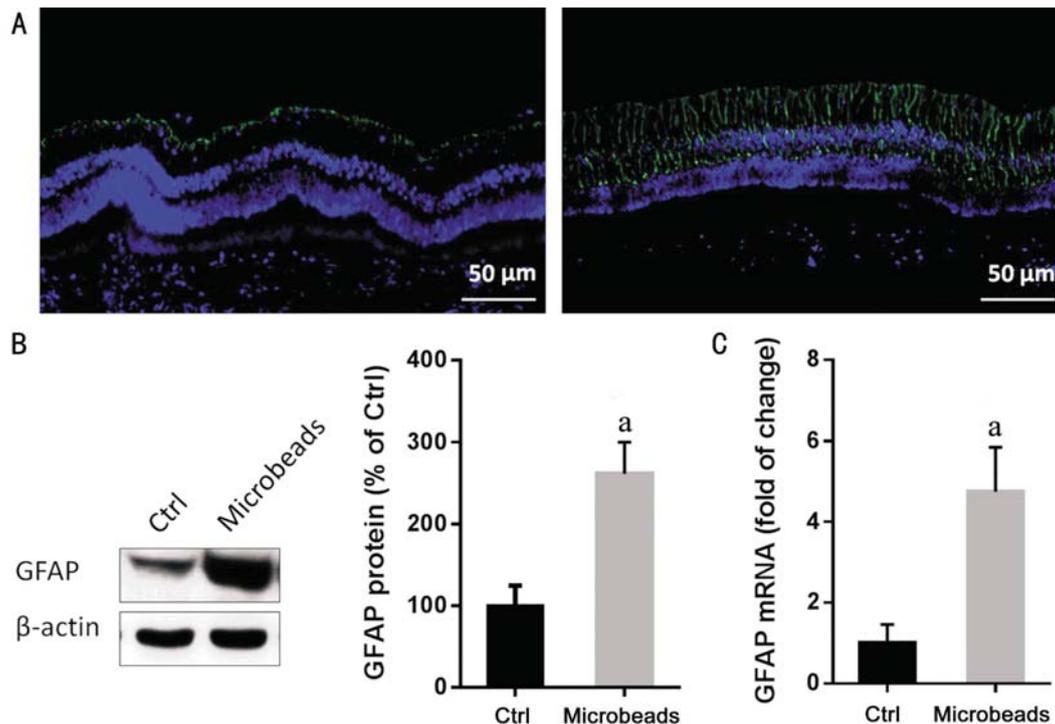


Figure 6 Increased expression of GFAP following the repeated injection of microbeads A: Immunofluorescence image illustrated the expression of GFAP (green) in the retina from the rabbits receiving two injections of PBS or microbeads. Sections counter stained with DAPI (blue). Scale bars: 50 μ m; B: The representative blot shows Western blot analysis from retina. The protein level of GFAP evaluated by western blot ($n=6$ /group); C: The mRNA level of GFAP evaluated by qPCR ($n=6$ /group). ^a $P<0.05$.

Table 1 The expression of GFAP mRNA in the retina of rabbit receiving the microbead injection

Injected group	Average C_T	ΔC_T	$\Delta\Delta C_T$	RQ
PBS	16.84 \pm 0.31	3.40 \pm 0.46	0.00 \pm 0.46	1.00 \pm 0.46
Microbead	13.82 \pm 0.27	1.21 \pm 0.39	-2.15 \pm 0.29	4.74 \pm 1.10 ^a

C_T value represents the number of qPCR cycles when the fluorescence reaches the threshold; $\Delta C_T = C_T$ value of GFAP - C_T value of β -actin; $\Delta\Delta C_T = \Delta C_T$ (microbead-injected) - ΔC_T (PBS-injected); RQ: Relative quantification = $2^{-\Delta\Delta C_T}$.

IOP value, and the significant IOP elevation was observed at day 3, which is consistent with the previous study^[19].

The angular meshwork and the aqueous plexus in the angular region of rabbits respectively correspond to the trabecular meshwork and the Schlemm's canal in that of humans. Therefore, the outflow pathway for aqueous humor in rabbits is similar to that in humans. That is, the aqueous humor in rabbits flows through the angular meshwork into the aqueous plexus and through the trabecular meshwork into Schlemm's canal in humans^[33-34]. Meanwhile, it is much more convenient to operate on the bigger size of rabbit eyeball compared to rats or mice and rabbits are low cost compared to primates, but current validated methods for inducing glaucoma in rabbits have some drawbacks. The longest IOP elevation was reported in injection of α -chymotrypsin into the posterior chamber, lasting for one year^[5], and injection of betamethasone subconjunctivally also achieved the consistency and robustness of the IOP response^[12]. However, the prolonged topical corticosteroid treatment required to achieve glaucoma led to cataracts and corneal ulcers^[3], while the α -chymotrypsin model caused significant adverse effects such

as bullous keratopathy, iris and ciliary body atrophy^[15]. In the laser-induced glaucoma models, the IOP elevation lasted for a few weeks but it was difficult to create a successful model due to the unique structure of narrow iridocorneal angle, which prevented access of the laser beam to angular trabecular meshwork, resulting in damage to ciliary body and severe inflammation^[35]. Compared with these methods, the microbead-injected model induced consistent and robust IOP elevation up to 8wk, which is easy to create without obvious ocular structure damage or inflammatory responses.

In this rabbit model, it has been demonstrated that optic nerve degeneration and axon loss have emerged similarly in rat or mice model^[19]. The optic nerve degeneration exhibited as axon swelling, disruption of axon fascicles, increases in connective tissue septa and lower axon numbers by IOP elevation. There was a statistically significant difference in the axon density between the PBS-injected group ($52.65 \pm 4.83 \times 10^4$ axons/mm²) and OHT group ($27.82 \pm 2.54 \times 10^4$ axons/mm²) ($P<0.05$). The extent of axonal loss was determined to be 47.16% \pm 2.35% in the OHT group compared with control group.

Glial cells activation, which is characterized by cellular hypertrophy and increased expression of GFAP, is a universal response in retinal pathologic states, including glaucoma^[24]. The previous study in different animal models^[29-31] and glaucomatous human donor eyes^[32] indicated that glial cell activation was a prominent feature of glaucomatous retina, and this microbead-injected model showed the hypertrophic morphology and increased GFAP immunostaining in retinal astrocytes and Müller cells in OHT eyes compared with PBS-injected eyes, providing a significant evidence for simulating glaucomatous changes successfully.

In summary, we have established a simple strategy to induce an IOP elevation, which is accompanied with the glaucomaous changes in both the optic nerve and retina by performing two separate intracameral injections of microbeads in rabbits. GFAP was identified in this research as a suitable and practical index for assessing of neuronal injury associated with IOP elevation, though the precise mechanism and role of GFAP in glaucoma requires a further attention. Development of this method offers a novel rabbit model of glaucoma employed as a powerful model system for the preclinical testing of drug therapeutics for the managing of glaucoma. However microbeads are required for repeated injection into the anterior chamber of rabbits for the purpose of consistent IOP elevation, and a further improvement is still necessary for this model in the future.

ACKNOWLEDGEMENTS

The authors appreciate Jeroen van Suylichem, Lei-Lei Tu and Xiao-Li Shen for critical review of the manuscript.

Foundations: Supported by Shenzhen Science and Technology Innovation Committee in China (No. JCYJ20120831154554508; No. JCYJ20140415174819509; No. GJHZ20160229170608241); Medical Science and Technology Research Fund Project in Guangdong Province (No. A2015315).

Conflicts of Interest: Zhao J, None; Zhu TH, None; Chen WC, None; Peng SM, None; Huang XS, None; Cho KS, None; Chen DF, None; Liu GS, None.

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