Int J Clin Exp Med 2022;15(9):272-281
www.ijcem.com /ISSN:1940-5901/IJCEM0143927

Original Article

Establishment of a rat model for chronic ocular hypertension by transplanting conjunctival fibroblasts into the anterior chamber

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Received May 5, 2022; Accepted August 10, 2022; Epub September 15, 2022; Published September 30, 2022

Abstract: This study aimed to establish a novel animal model of chronic ocular hypertension (COH) by introducing conjunctival fibroblasts (CFs) into the anterior chamber (AC) of the eyes of rats. Anesthetized Sprague-Dawley rats received the transplantation of cultured CFs into the AC in their right eyes. Proliferated CFs induced occlusion of the anterior angles and blocked the aqueous drainage. Intraocular pressure (IOP) was monitored for at least 12 weeks using a TonoLab under anesthesia. The IOP increased from 11.15±0.38 to 39.19±2.33 mmHg 3 days after CF injection and remained elevated above 20 mmHg for at least 12 weeks. The retinal ganglion cells (RGCs) were labeled with fluorogold retrograde staining and were counted in retinal flat whole mounts at different time points. The RGC count in the COH eyes decreased in a time-dependent manner by 11.66%, 32.87%, 56.24%, and 67.38% at weeks 2, 4, 8, and 12, respectively. The anterior part of the eye and the optic disc were assessed by hematoxylin-eosin staining. Cupping of the optic disc was noticed at week 4, 8, and 12 after the CF injection. Transmission electron microscopic examination showed a significant reduction in the axon bundles of the optic nerve compared with the untreated eyes. It was concluded that the introduction of CFs into the AC could block the aqueous drainage and induce COH in rat's eyes, resulting in the loss of RGCs and optic nerve degeneration over time, which might mimic the development and progression of chronic glaucoma in humans.

Keywords: Chronic ocular hypertension, conjunctival cell, glaucoma model, rat, retinal ganglion cell

Introduction

Glaucoma is still a leading cause of irreversible blindness all over the world, characterized by the progressive loss of retinal ganglion cells (RGCs) and visual field damage [1, 2]. Abnormally high intraocular pressure (IOP) plays a primary role in the development and progression of glaucoma [3], and IOP is still the only modulable risk factor for glaucoma treatment. Detailed studies on the molecular mechanisms of glaucoma in humans are complicated by accessibility to human donor eyes with glaucoma histories, limitations on death to preservation time, and also that the majority of glaucoma donor eyes are at late stages of the disease, etc. [4]. Animal models are among the most useful tools for understanding disease pathology and developing treatment interventions.

To date, many rodent models have been reported to mimic the natural cause of glaucoma by increasing a sustained IOP increase over time, including laser photocoagulation of the trabecular meshwork (TM) [5-8], injection of hypertonic saline into the episcleral veins [9], cauterization of the episcleral veins [10-13], injection of different substances into the anterior chamber (AC) to block the aqueous drainage [14-16], and circumlimbal suture to induce ocular hypertension [17-20]. Most of these models are able to induce an increase in IOP within a few days, but are maintained for only a couple of weeks after the procedure. This relatively short time of

IOP increase may not be long enough to mimic the chronic glaucomatous changes in the eyes of the human being. Novel ideal animal models are essential to elucidate the pathogenesis of glaucoma and develop treatment measures.

The present study aimed to establish a novel chronic ocular hypertension (COH) model by injecting cultured conjunctival fibroblasts (CFs) into the AC of rat eyes. The change in the IOP, morphology of the AC angle and optic nerve, and number of RGCs were studied.

Materials and methods

Animals

In accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the study protocol was approved by the experimental animal ethics committee at Xiamen University, China. Eighty Sprague-Dawley male rats (180-200 g, 8-10 w old) obtained from Shanghai Laboratory Animal Center, Shanghai, China) were maintained on 12-h light-dark cycles. They were given food (standard lab chow) and water *ad libitum*. They were intraperitoneally injected with chloral hydrate (10 mg/100 g) for general anesthesia. Animals were randomly chosen for histological assessment and RGC counting at 2, 4, 8, and 12 w after sustained IOP increase had been observed for 1 w. Topical instillation of 0.5% proparacaine (Alcon, TX, USA) was applied to the eyes when necessary.

Preparation and injection of CFs

Under sterile conditions, a piece of conjunctival tissue with the size of 2 × 3 mm^2 taken from the eye of a green fluorescent protein (GFP)-transgenic Sprague-Dawley rat was dissected and used for cell culture. The tissue was digested in collagenase IV at 37°C for 2 h to harvest the CFs. The cells were then cultured in Dulbecco's minimum essential medium (5% CO_2, 37°C) with 10% fetal bovine serum (FBS). They were repeatedly cultured every 3 days when full confluence was achieved.

The CFs were trypsinized, and the cell suspension was prepared in Dulbecco's modified Eagle's medium with 10% FBS (1.0 × 10^7 cell/mL; 1 mL). After general anesthesia with 10 mg/100 g chloral hydrate in the rats, the CF suspension was injected into the AC of the right eyes of the rats through a 10-μL syringe (Agilent, USA), and the left eyes were left untreated as controls. Topical anesthesia with 0.5% proparacaine (Alcon, Fort Worth, TX, USA) was applied in case of necessity. After CF injection, the rat eyes were treated with ofloxacin ointment once daily for 3 days. COH induction was regarded to be successful after the IOP doubled to the baseline and lasted for at least 1 w.

IOP measurement

The IOP was measured using a TonoLab (Tiolat, Helsinki, Finland) tonometer under anesthesia. Averaged IOP readings from five measurements were recorded. The IOP was monitored daily for at least 12 w after CF injection.

Histological assessment of the ocular tissue

After the rat was sacrificed with an overdose intraperitoneal injection of 10 mg/100 g chloral hydrate, the eyeball was enucleated and immediately frozen in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). The eyeball was sectioned along the meridian with a thickness of 10 μm to show the histological changes in the anterior part of the eyes. Tissue preparations were stained with hematoxylin-eosin (H&E) and viewed under an optical and/or fluorescence microscope. The anesthetized rats were then perfused intracardially with cold 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.1 mol/L, pH7.4) to show the histopathological change in the optic nerve after COH. The eyeballs with intact optic nerves were quickly excised and fixed in 4% paraformaldehyde with 0.1 mol/L PBS for 2 h. The corneas and lenses were dissected, and the rest of the globe was left in the same fixation solution overnight. Tissue preparations were dehydrated with graded ethanol, embedded in paraffin, and sectioned at 5 μm thickness.

Retrograde labeling of RGCs in the rat retina and RGC count

Retrograde staining of RGCs was performed as described previously [21]. In brief, the rat was mounted on a stereotactic apparatus (RWD Life Science, Shenzhen, China) with the skin over the skull incised. The parietal bone was perforated using a dental drill, and the brain surface was exposed. A fluorogold (4%) (Fluorochrome

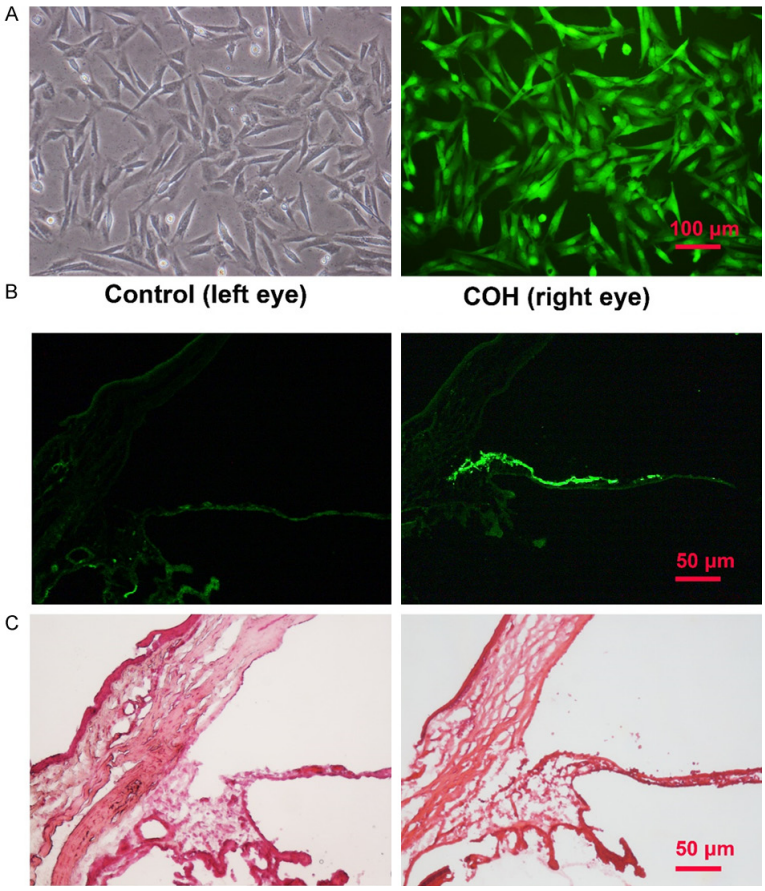

Figure 1. Eyes with chronic ocular hypertension (COH) 1 week after conjunctival fibroblast transplantation. A. Fibroblasts cultured from the conjunctiva of a green fluorescent protein (GFP)-transgenic Sprague-Dawley rat. B. Cross-section of the angle under a fluorescent microscope. Left: control; right: COH eye. C. H&E staining of the rat angle structure. The angle was occluded by the proliferated fibroblasts.

LLC, CO, USA) injection (3 μl each) was given at the points 5.0 mm caudal to the bregma and 1.0 mm lateral to the midline on both sides with a depth of 5.00 mm from the surface of the skull. One week after the fluorogold injection, the rat was sacrificed. The eyes were excised and fixed in 4% paraformaldehyde in PBS for 1 h in the dark at 4°C. The anterior part of the globe was dissected, and the eyecups were again fixed for 30 min. Four cuts were made radially on the sclera, and the retinas were dissected with care from the underlying retinal pigment epithelium. The retinas were then mounted flatly onto glass slides in the dark at 4°C and inspected under a fluorescence microscope (Leica, DM2500, Wetzlar, Germany). RGCs on each quadrant labeled using fluorogold were counted in a masked fashion by another researcher at three distances (1/6, 3/6, and 5/6) radially from the optic nerve. RGC count per image was determined by two masked observers.

Ultrastructural changes in the TM and the optic nerve

Twelve weeks after CF injection, the rats were anesthetized deeply with 10 mg/100 g chloral hydrate and transcardially perfused with 50 mL of 0.9% saline followed by 4% paraformaldehyde. The eyeballs with intact optic nerves were enucleated and fixed in a 0.1 mol/L PBS containing 1.5% paraformaldehyde and 2% glutaraldehyde (pH7.6), osmicated with 1% osmium tetroxide, and embedded in Epo-Araldite resin (Ladd Research Industries, VT, USA). Semi-thin sections of the optic nerves were stained with 1% *p*-phenylenediamine (P-6001; Sigma, MO, USA) to identify myelinated axons. The ultrastructural changes in the TM and the optic nerve were assessed under a transmission electron microscope (TEM) (JEM-2100, JEOL Ltd. Japan).

Statistical analysis

Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc., CA, USA). The paired *t*-test was used when comparing data sets collected from the same group of animals. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons were performed between and within groups. A *p* value less than 0.05 was considered to be statistically significant.

Results

Obstruction of the AC angle by the fibroblasts

The CFs were determined morphologically during the culture period (**Figure 1A**). After cell injection into the AC, cell proliferation was observed on the iris surface and angle space (**Figures 1B**, **1C** and **2**). Obstruction of the TM by the cells was seen in the tissue sections under the microscope for at least for 4 weeks

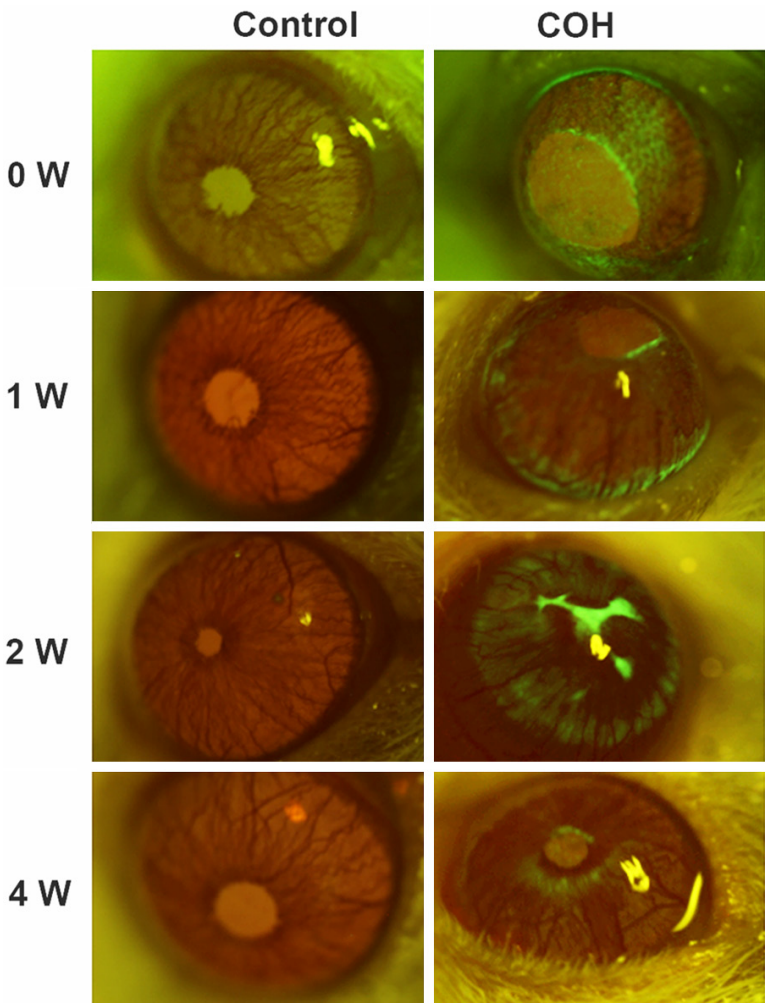

Figure 2. Conjunctival fibroblasts from a GFP-transgenic Sprague-Dawley rat under a fluorescent microscope after being injected into the anterior chamber of the rat. COH, chronic ocular hypertension.

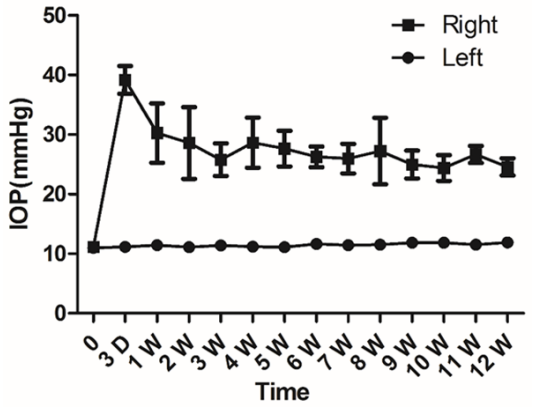

Figure 3. Intraocular pressure (IOP) recording on the right (chronic ocular hypertension, COH) and left (control) eyes of rats. An IOP elevation was observed in all rats after the injection. The average IOP on the COH eyes peaked at 39.19±2.33 mmHg on the third day after the injection, which remained elevated for at least 12 weeks (n = 20).

(**Figure 2**). Fibrous synechiae of the lens and iris were noted 1 week after the injection of CFs. Corneal edema was seen in some eyes with an abnormally high IOP (**Figure 3**).

IOP increase and size change of the eyes after CF injection

An increase in IOP was observed with a range of 17-46 mmHg on the injected eyes, while the IOP on the eyes without CF injection remained normal (10-15 mmHg). The mean IOP of the COH eyes was 39.19±2.33 mmHg on the third day after the injection, remained elevated for at least 12 weeks, and was 24.58±1.45 mmHg at week 12 after the injection (**Figure 3**). The average IOP of the control eyes was not significantly different ($P > 0.05$) during the observation period. The enlargement of the eyeball and atrophy of the optic nerve were observed at week 12 (**Figure 4**).

RGC count

The change in RGC count after CF injection is shown in **Figure 5**. The RGCs in the COH eyes decreased significantly from week 2 after CF injection. However, in the untreated left eyes, the RGC count did not show any significant change at any time point (**Figure 5**). The average rate of RGC loss in the COH eyes from baseline was 11.66%, 32.87%, 56.24%, and 67.38% at weeks 2, 4, 8, and 12, respectively.

Morphological change in the optic nerve/disk and the TM after CF injection

The enlargement of optic disk cupping was observed at weeks 4, 8, and 12 (**Figure 6**). Under the TEM, the number of axon bundles of the optic nerve (41.6±6.09 per mm^2) was significantly ($P < 0.001$) reduced compared with untreated eyes (132.7±7.1 per mm^2) 12 weeks after CF injection. In addition, the axon bundles in the COH eyes were surrounded by glial tissues (**Figure 7A**). In the TM after cell injection,

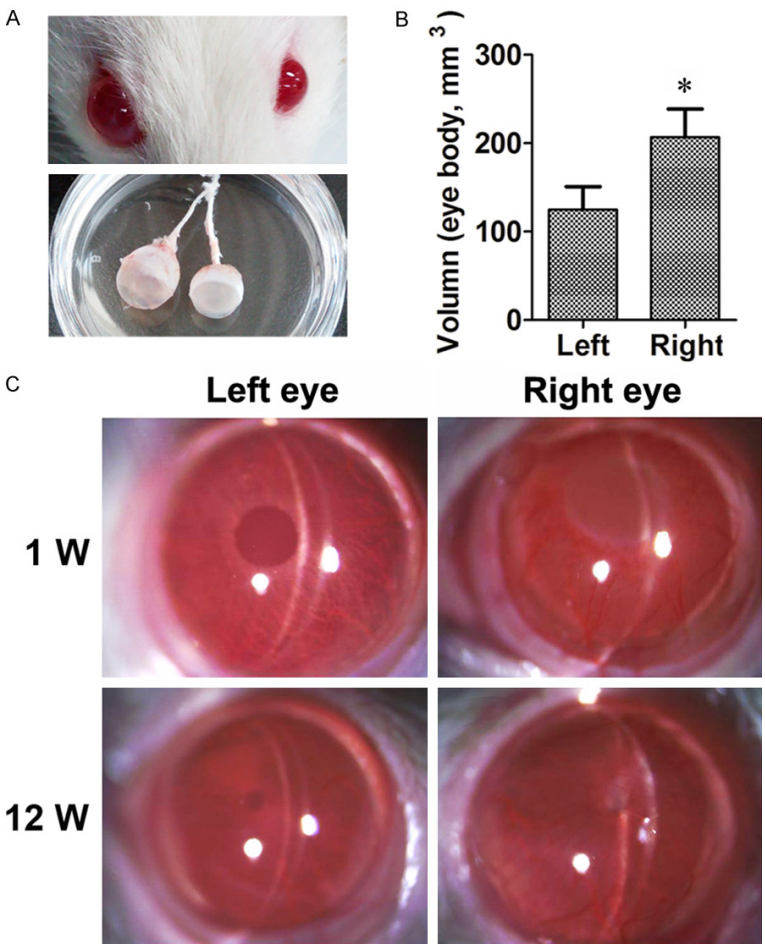

Figure 4. Change in the size of the COH eyes and control eyes 12 weeks after conjunctival fibroblast injection. COH, chronic ocular hypertension. *$P < 0.01$, vs. left eyes.

the collapse of the TM was seen with significant fibrous proliferation (**Figure 7B**).

Discussion

In the present study, a novel animal model of COH was established by injecting CFs into the AC of the rat eyes. The GFP-labeled CFs were used to demonstrate the fate of the injected cells (**Figure 1A**). As shown in the histological sections, proliferated CFs covered the surface of the AC angles (**Figure 1B** and **1C**). After the injection, the IOP was significantly elevated in the treated eyes and peaked at 39.19±2.33 mmHg on day 3 after the injection. However, the IOP in the untreated eyes remained unchanged compared to the baseline. In the present study, without exception, all injected eyes showed a significant increase in IOP from baseline, which lasted for at least 12 weeks. This success rate was significantly higher than those reported by other studies of rat COH models.

Rodent models of diseases are particularly advantageous for their excellent availability, relatively low cost, short lifespan, and compliance with experimental and genetic operations [4, 22]. Many rodent models have been developed to mimic the natural cause of chronic glaucoma under an abnormally high IOP [5-17], however, these animal models of glaucoma have varied limitations that hinder their use in scientific research [4]. Most of these models only induce an IOP increase within a short period ranging from a few days to a couple of weeks, such as the methods of injection of hypertonic saline into the episcleral veins [9], cauterization of the episcleral veins [10-13], injection of different substances into the anterior chamber [14-16], and circumlimbal suture [17], et al. Repeat manipulation of the procedures is necessary to maintain the IOP at an abnormally high level, this may introduce extra injury to the animal and alter the natural course of the disease. In animal models of laser photocoagulation of the TM [5-8], costly equipment particularly designated for laser treatment is needed. For 77 of the 80 rats studied in the present study, successful induction of sustained IOP elevation was seen after a single CF injection, with an additional injection needed in only 3 rats. The injury introduced to the animals during the procedure was minimized and prevented the rats from additional damage to the eye other than glaucoma.

There exists a report in the literature describing the establishment of a ferret glaucoma model by injecting CFs into the AC, the authors observed a sustained IOP increase for 13 weeks [23]. Consistent with their findings, in the present study, a stable and sustained IOP

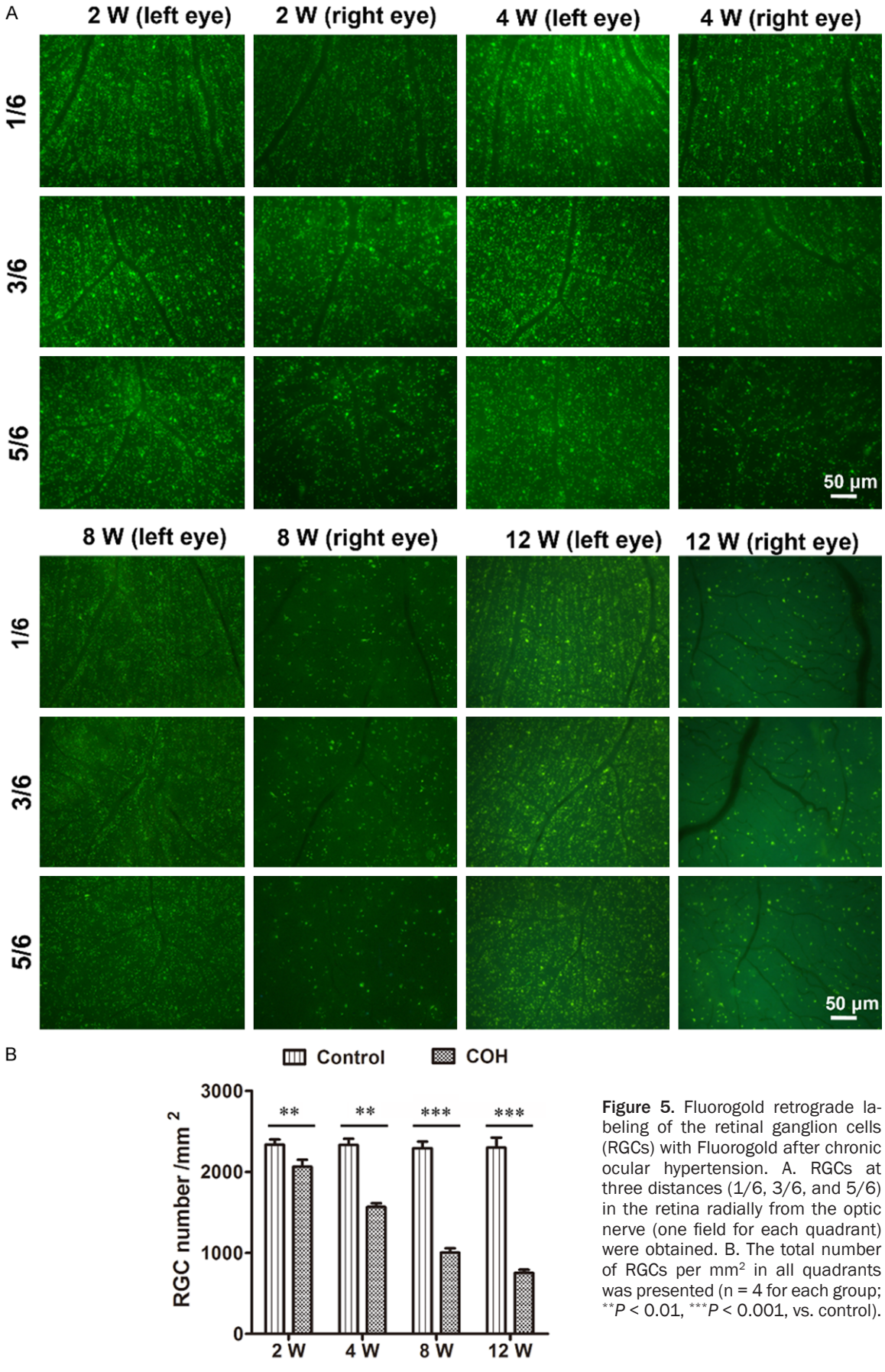

Figure 5. Fluorogold retrograde labeling of the retinal ganglion cells (RGCs) with Fluorogold after chronic ocular hypertension. A. RGCs at three distances (1/6, 3/6, and 5/6) in the retina radially from the optic nerve (one field for each quadrant) were obtained. B. The total number of RGCs per mm^2 in all quadrants was presented (n = 4 for each group; $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control).

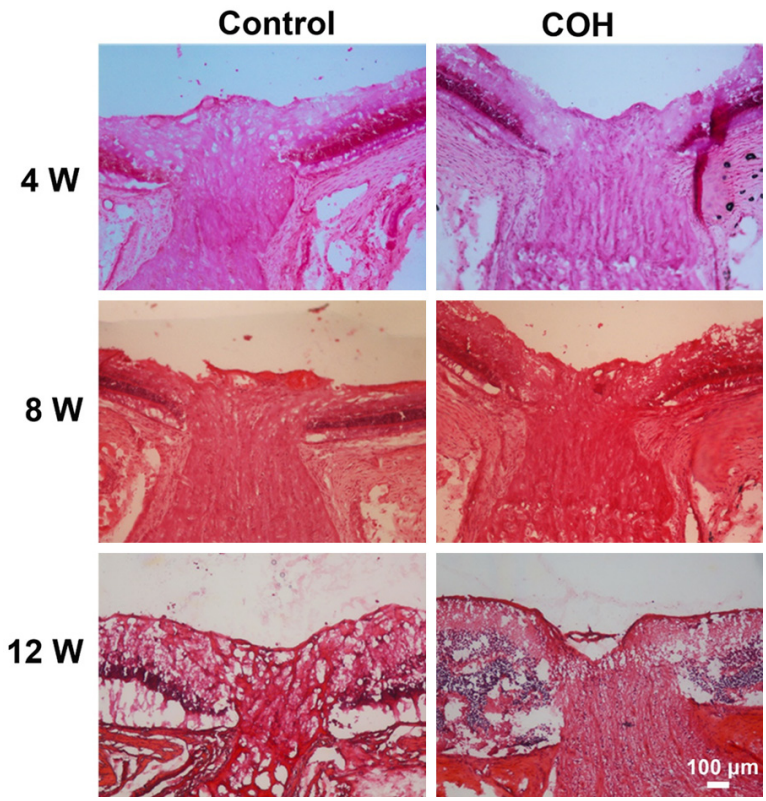

Figure 6. H&E staining of the optic nerve head and the optic disk of COH control eyes. Enlargement and deepening of the optic nerve disk cupping were observed. COH, chronic ocular hypertension.

rise was noticed for at least 12 weeks (**Figure 3**). Eyeball expansion was observed as a result of a sustained increase in IOP (**Figure 4A**). The atrophy of the optic nerve was also observed, particularly in the part near the chiasma opticum (**Figure 4A**). Cupping of the optic disc (**Figure 5**), as well as the degenerative structural change of the optic nerve (**Figure 7B**), suggested typical pathological alterations due to glaucoma. As a consequence, ultrastructural changes including the failure of intra-axonal transport, subsequent loss of RGC presynaptic active zones and axon terminals in central projection sites, and degeneration of target neurons may account for the functional loss in seen glaucoma.

Although ischemia-reperfusion injury (IRI) may partially account for the RGC loss and retinal damage induced by the abnormally elevated IOP, as reported in many other studies [24-27], the animal model presented in our study is still different from IRI. Firstly, in our study, the IOP peaked at 39 mmHg 2 days after CF injection and remained elevated above normal levels afterward. This IOP change profile was inconsistent with those reported in other rat models of chronic glaucoma [15, 23] and much lower than that in some IRI animal models, where the IOPs are usually elevated to 110-120 mmHg and last for a few hours [21, 22]. Furthermore, as described above, the change in the size of the eyeball, the cupping of the optic nerve head, together with the loss of retinal ganglion cells, are rather typical manifestations of a high IOP-induced chronic glaucoma than IRI-induced retina/optic nerve change.

Previous rodent models of glaucoma/ocular hypertension have shown an association between IOP increase and characteristic RGC damage [28-31] and dysfunction [31-34]. In the present study, the retrograde fluorogold labeling of the RGCs was performed at weeks 2, 4, 8, and 12 after the injection to evaluate the loss of RGCs induced by the elevation of IOP. The result of cell count revealed that the viable RGCs in the COH eyes markedly decreased in a time-dependent manner. This observation confirmed the success of the COH model establishment which may be used as an effective and reliable model for glaucoma research. The mechanisms underlying the IOP increase in the present rat model are complex. As shown in histological tissue preparations, the proliferation of CF in the AC occluded the angle through which the aqueous outflow leaves the globe (**Figures 4C** and **7A**). In addition, the membrane formed above the pupil may induce a pupil block, which may subsequently promote the closure and occlusion of the AC angles. Different from some other rodent glaucoma models where multiple injections or laser treatments are necessary to maintain an elevated IOP, in our study only a single injection of cultured CF was needed to induce a stable IOP

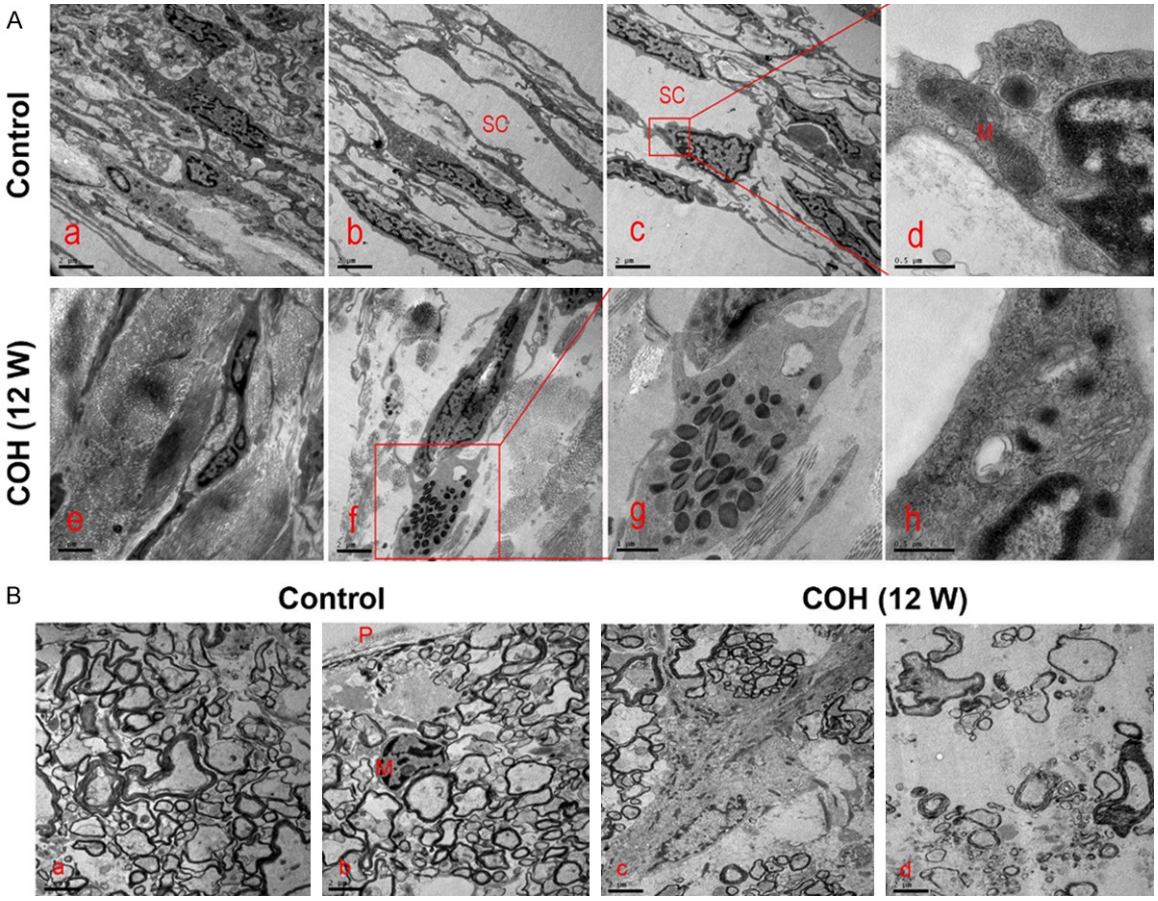

Figure 7. Ultrastructural change in the trabecular meshwork (TM) and optic nerve after conjunctival fibroblast injection. A. Collapse of the TM with significant fibrous membrane formation. Destruction of the nucleus was observed inside the TM cells with the deposition of pigment particles. B. Loss of axon bundles of the optic nerve 12 weeks after chronic ocular hypertension (COH) induction.

increase over a long period of time, preventing additional injury to the eyeball.

The present study demonstrated a simple and feasible method to establish a rat model of COH/glaucoma. Sustained IOP over time with no need for multiple injections or manipulation is the biggest advantage of this animal model, which mimics the chronic nature of glaucoma. This also provides an essential advantage in the research that needs to be monitored over a long period. Nevertheless, this model had some weaknesses. First, transplantation of cultured CFs might induce an intraocular inflammatory reaction and inevitably influence the tissue metabolism inside the eye. Second, the fibrous membrane covered the pupil and hindered direct inspection of the posterior part of the globe, rendering it difficult or even impossible to image the retina and the optic nerve head. To prevent this from occurring, interventions to reduce the inflammation reaction in the anterior chamber, together with the mydriatic treatment may be of importance.

In summary, transplantation of cultured CFs into the AC to induce COH in rat eyes is minimally invasive and easily performed. This approach successfully occluded the angle and obstruct aqueous outflow, resulting in an IOP elevation for at least 12 weeks. RGCs in the COH eyes decreased with time, accompanied by deepening of the optic disc cupping, loss of RGCs, and the axon bundles of the optic nerve. This animal model mimicked the pathophysiological process of glaucoma with a high success rate which may facilitate future research to understand and treat glaucoma. In future study, we need to overcome the disadvantages of this COH animal model by reducing the

inflammation of the anterior chamber to mimic more precisely the natural course of glaucoma in humans. Mechanisms underlying the IOP rise, as well as the pathological changes in the retina, optic nerve, and the visual pathway in the central nervous system will be investigated. Reactions of this animal model to surgical and drug treatments are also of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81570844), Natural Science Foundation of Fujian Province (No. 2011D001), Medical Innovation Program of Fujian Province (No. 2011-CXB-47); Huaxia Translational Medicine Youth Foundation (No. 2017-A-00301) and the Xiamen Science and Technology Program Guiding Project (No. 3502Z20189033).

Disclosure of conflict of interest

None.

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