**TITLE:**

**Study of glaucomatous pathology in an animal model**

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**SHORT ABSTRACT:**

In this paper, we present a mouse model of glaucoma that depicts the features of the glaucomatous pathology in humans and demonstrates how it might be used for preclinical studies.

**LONG ABSTRACT:**

Due to iris pigment dispersion (IPD) and iris stromal atrophy (ISA), the DBA/2J mouse is a model of ocular hypertension and retinal ganglion cell (RGC) degeneration. These mice present increases in intraocular pressure (IOP) typical in many glaucoma types. This increase correlates with the death of the RGCs. The RGCs can be investigated at different ages through retrograde tracing with an analogue of fluorogold, hydroxystilbamidine methanesulfonate (OHSt), applied on the superior colliculi. Whole-mount retinas were processed to quantify the population of RGCs identified by fluorogold tracing and were counted using image analysis software. An isodensity contour plot was generated for each retina. These progressive processes could also be followed by means of electroretinographic recordings that clearly correlated with morphological changes (loss of RGCs). The DBA/2J mouse presented a rise in IOP which started at 9 months and became maximal at 12 months, with an increase of 70% above the initial IOP. Quantification of the total number of RGCs identified by OHSt showed a clear increase in cell death as the pathology advanced; this was also correlated with a decrease in the in pSTR electroretinographic recordings. Our results indicate the existence of a correlation between rises in IOP, retinal function impairment, and RGC loss. This functional and morphological analysis allows for the reliable assessment of the progression of the disease and permits the use of this animal as a preclinical model for the investigation of potential antiglaucomatous drugs.

**INTRODUCTION:**

Glaucoma consists of a group of optic neuropathies that are characterized by the progressive loss of retinal ganglion cells (RGCs)1-3, with a concomitant loss of visual function that may result in permanent blindness2. This pathology is often associated with an abnormal increase in the intraocular pressure (IOP), the only major risk factor that can be modified with medical or surgical treatment4-7.

Patients with IOP values above 21 mmHg are further examined with ophthalmological tests for the presence of typical optic disk abnormalities and thinness of the retinal nerve fiber layer. Any loss of visual function is examined with visual field tests (perimetry) or electroretinography in order to fully confirm the existence of the pathology. When glaucoma is diagnosed, there are several therapeutic options, pharmacological or surgical, that can be implemented to avoid further progression of the disease.

Several glaucoma animal models have been developed to mimic some of the features of this pathology. A popular group of animal models is based on the elevation of the IOP. This may be achieved with the application of substances such as corticoids, alpha chemotrypsin, phenol8-12, or hyaluronic acid, which increase the volume of the aqueous humour. Alternative strategies, such as cauterization of the episcleral or perilimbal veins or photocoagulation of the trabecular meshwork, diminish the aqueous humor outflow. Other, more dramatic models, such as optic nerve axotomy, may also contribute to the investigation of injuries associated with RGC loss.

Despite the existence of several animal models for experimental glaucoma13,14, there was a lack of an animal model that spontaneously developed a glaucomatous pathology. Since 1927, the mouse strain DBA/2J has been used in laboratories for many research purposes, including cardiovascular biology, neurobiology, and sensorineural research. It was only recently realized that this strain develops a spontaneous exfoliative glaucoma. Although this type of glaucoma is not the most representative of that in humans, its development in mice mimics the progression of human glaucoma.

DBA/2J presents a gradual increase in IOP, which is maximal between the 9th and 12th month of age. This rise in the IOP is mainly due to the iris pigment dispersion in the eye anterior chamber produced by an iris atrophy. The rise in IOP might be the key factor that produces retinal cell damage in this animal model. Retinal cell death concomitantly produces a visual dysfunction that can be recorded by electroretinography. In conclusion, the rise in IOP, the retinal damage, and the retinal loss-of-function are three hallmarks of the glaucomatous pathology in humans that are measurable in the DBA/2J mouse model.

In the present work, we introduce how to measure three aspects of glaucoma: IOP, retinal cell death, and changes in the retinal electrophysiology. Altogether, it will be possible to study how glaucoma progress in these animals. Moreover, this animal model will be helpful in the investigation of the major challenges of glaucoma treatment: the search for hypotensive compounds and the exploration of compounds with retinal neuroprotective properties.

**PROTOCOL:**

All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

NOTE: Experiments were performed on adult female C57BL/6J (n=8) and DBA/2J (n=8) mice. Mice were housed (1-4 mice per cage) in temperature- and light-controlled rooms maintained according to a 12-hr light/dark cycle. All animals were fed *ad libitum*. DBA/2J and C57BL/6J mice were studied at 3, 6, 9, 12, and 15 months of age. The experiments were performed on female DBA/2J and C57BL/6J mice since IOP increases earlier in females and remains elevated longer15.

**1. Assessment of intraocular pressure measurements**

1.1 Ideally, perform the IOP measurements at the same time of day to avoid fluctuations due to circadian rhythms16,17.

1.2 Anesthetize the mice in accordance with institutional guidelines. Inject mice with a mixture of ketamine (95 mg/kg) and xylazine (5 mg/kg) through an intraperitoneal (i.p.) injection.

1.2.1. Confirm anesthesia effectiveness with a gentle toe pinch, which should not break the skin or cause any deep tissue damage, or by corneal reflex (touching the edge of the cornea with a cotton tip).

NOTE: Any observed movement (withdrawing the paw or movement of the eyelids) indicates that the animal is not sufficiently anesthetized for surgery.

1.3 Bring the tonometer close to the mouse’s eye, fixing the tonometer with hands and/or to some solid object. The distance must be 1-4 mm from the tip of the probe to the cornea of the eye. Ensure that the tip of the probe hits perpendicularly to the central cornea. Measure both eyes of each animal at 3, 6, 9, 12, and 15 months of age.

NOTE: To perform intraocular pressure measurements, a non-invasive rebound tonometer4,14,18,19 was used, and readings were taken just after the application of anesthesia20.

1.4 Collect six consecutive measurements for each reading and repeat it three times to get a final IOP value14,21.

**2. Electroretinographic recordings**

2.1 Dark-adapt the mice overnight. Carry out all manipulation procedures under dim red light (l > 600 nm).

2.2 Calibrate the light intensity with a dual-biosignal generator device specifically adapted for ERG responses in order to ensure consistent recording parameters across the different ages.

2.3 Anaesthetize the mice with an i.p. injection of a solution of ketamine (95 mg/kg) and xylazine (5 mg/kg).

NOTE: Confirm anesthesia effectiveness (see 1.2.1).

2.4 Dilate the pupils by applying a topical drop of 1% tropicamide in the right eye of the experimental animal five min before ERG testing.

2.5 Proceed to situating the electrodes:

2.5.1 Apply a topical non-allergenic ionic conductive drop of methylcellulose to each eye before situating the corneal electrode.

NOTE: This approach is necessary to minimize corneal desiccation and maximize conductivity of the generated response.

2.5.2 Keep the working electrode, a mouse Burian-Allen lens electrode, in contact on the corneal surface covered with methylcellulose. Place the reference electrode in the mouth. Place a needle subcutaneously at the base of the tail as the ground electrode.

2.6 Maintain the mice on a circulating warm water pad at 37 ºC. Place them in a Ganzfeld stimulator to ensure consistent experimental conditions.

2.7 Stimulate flash-induced ERG responses with a Ganzfeld dome light source, which ensures homogeneous illumination of the retina and emits a wide range of light intensities.

NOTE: Using a commercial amplifier, amplify the electrical signals generated in the retina and filter the bands between 0.3 and 1,000 Hz. Digitalize the electrical signals at 20 kHz using a data acquisition board and display them on a PC computer monitor. Record the scotopic threshold response (STR) by stimulating the retina with light intensities ranging between 10-5 and 10-2 cd s m-2 in the right eye. Average a series of ERG responses (20 ERGs for each trace) at each light intensity after adjusting the time interval between flashes to ensure complete recovery of the response (10 sec for the dimmest stimulus intensities and 30 sec for the strongest stimuli).

2.8 Analyze the different recordings of the amplitudes of the different waves according to the criteria established by the International Society for Clinical Electrophysiology of Vision (ISCEV).

NOTE: This ERG protocol has been previously used by several authors as a functional test for the study of retinal ganglion cell population5,6,22-27.

2.9 Maintain the mice on a heating pad to assure normal body temperature at 37 °C during recovery from the induced anesthesia.

2.10 Apply ocular ointment containing neomycin and prednisone to prevent corneal desiccation.

**3. Retrograde labeling study**

3.1 Anesthetize the mice with an i.p. injection of ketamine (70mg/kg) and xylazine (10mg/kg). Confirm anesthesia effectiveness (see 1.2.1). Apply sterile eye lubricant ointment to prevent drying of the corneas during surgery.

3.2. Shave the head with an electric clipper equipped with a surgical blade. Remove the clipped hair from the animal using a vacuum-system or tape. Disinfect the operation area with 10% povidone iodine solution.

NOTE: Retrograde labeling was performed as described elsewhere4,6,26-30.

3.3. Cut the scalp along the midline with a #15 scalpel blade attach to a reusable scalpel blade handle. Expose the skull. Perform a bilateral parietal craniotomy. Use a dental drill to expose the dura mater, and cut it with fine forceps or spring scissors.

3.4. Carefully remove the brain cortex over the dorsal surface of the superior colliculi (SCi) using aspiration with a fine glass cannula attached to a vacuum pump. Operate under surgical microscope visual guidance.

NOTE: Aspirate the brain content until the SCi limits are observed under the microscope. Be careful not to touch the sinus

3.5. Place a small piece of gelatin sponge soaked in the tracer solution (10% hydroxystilbamidine methanesulfonate (OHSt) and 10% dimethyl sulfoxide (DMSO) in 0.9% saline), covering the entire surface of both SCi.

NOTE: Retinal ganglion cells (RGCs) are labeled retrogradely with the persistent marker OHSt**.** It is possible to identify RGCs due to the active retrograde axonal transport from the superior colliculi (SCi) when this compounds is applied to the SCi one week before animal processing4,6,28-30.

3.6 Close the skin with suture wound clips.

3.6.1. Leave the animals to recover in a breeding cage with flat paper bedding, and keep them warm with the aid of a heating pad. Monitor the animals and return them to the breeding house when they recover.

3.6.2. For five days, monitor the mice for general conditions and signs of infection after surgery.

**4. Immunohistochemical study**

4.1 Isolating the mouse retina

4.1.1 Anesthetize the mice and take the eye orientations. Place 6/0 suture silk through the ocular conjunctive on the superior pole of each eye to prepare retinal whole-mounts.

NOTE: The insertion point of the rectus muscle and the nasal caruncle were used as additional landmarks31.

4.1.2 Perfuse the mice transcardially with saline and 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2-7.4) at 4 °C41.

4.1.3 Enucleate and remove the cornea with curved tip forceps and sterile fine-angled dissecting scissors, following the protocol described by Mahajan and co-workers42. Cut around to the limbus. Move forceps and, with a slight back angle, pull up the cornea. Then, remove the ciliary body and lens with slight pressure on the eyecup.

4.1.4 Dissect out the mice retinas as flattened whole-mounts by making four radial cuts in the superior, inferior, nasal, and temporal retinal poles (the deepest in the superior pole). Carefully, remove the vitreous without damaging the retina by pulling it up with curved forceps located inside the posterior chamber.

4.1.5 Fix the retinas for an additional hour in the fixative solution (4.1.2), rinse them in 0.1 M PBS, mount them with the vitreal side up on subbed slides, and cover them with anti-fading mounting media.

4.2 Immunolabeling

4.2.1 Permeate the whole-mount retinas with PBS 0.5% Triton X-100 for 15 min at ‑70 °C, and then wash them in fresh PBS 0.5% Triton X-100 for 10 min.

4.2.2 Incubate the retinas in blocking buffer (PBS with 2% Triton X-100 and 2% normal donkey serum).

4.2.3 Incubate the whole-mount retinas, that were previously treated with OHSt5,6,14,29,30,32-34, in the Brn3a primary antibody (diluted 1:100 in blocking buffer) overnight at 4 °C.

NOTE: In order to verify RGC survival in the retina when the mice were sacrificed, Brn3a goat antibody is used since it labels a transcription factor specifically expressed in adult RGCs32,33,35,36.

4.2.4. Rinse the retinas three times in PBS.

4.2.5 Incubate for 2 hr at room temperature with the secondary antibody, diluted 1:500 in blocking buffer.

4.2.6 Finally, wash the retinas carefully in PBS and mount them with the vitreal side up on subbed slides. Cover them with antifading solution and place a cover glass onto the mounting medium. Seal with nail polish.

**5. Retinal whole-mount reconstructions**

5.1 Photograph the different groups of retinas under a fluorescence microscope equipped with an ultraviolet (BP 365/12, LP 397) and a rhodamine (BP 546/12, LP590) filter to observe the white-gold OHSt fluorescence and orange-fluorescent dye-conjugated antibodies, respectively. Analyze them.

NOTE: The microscope should be equipped with a digital high-resolution camera and a computer-driven motorized stage connected to an image analysis software through a microscope-controller module.

5.2. Photograph retinal multiframe acquisitions in a raster scan pattern, and capture the frames side-by-side with no gap or overlap between them with a 20x objective. Focus single frames manually before capturing each image, and then upload them into the image analysis program.

5.2.1 Define a scan area to cover the entire retina consisting of a matrix of m frames in columns and n frames in rows. Indicate the total number of frames in the scan area by frames in columns times frames in rows (m x n).

5.3 Capture 140 consecutive frames at a resolution of 300 dpi. Perform retinal whole-mount reconstructions following previously-described procedures5,6,14,28-31.

5.4 Measure the area of retinal whole-mounts on the high-resolution photomontage images of the complete retinas using a suitable software. Process reconstructed images with an image-editing computer software as needed to produce printouts.

**6. Morphometric analysis of retinal whole-mounts**

6.1 Process all images taken of the retinas with specific macros written in the image analysis program macro language that apply a sequence of filters and transformations in turn to each image of the stack.

6.2 Count the resulting cells and transfer the data to a spreadsheet for analysis. These subroutines have recently been described in detail6,14,28-30.

6.3 Analyze the distribution of RGCs using isodensity maps for each retina. Calculate the cell densities and represent them as a filled contour plot graph, following previously-described methods14.

NOTE: Every frame captured was divided into an equal number of 36 sampling areas of interest (AOI) for OHSt or Brn3a labeling. These AOI were automatically counted, and data were exported and saved to a spreadsheet software.

6.4 Represent these densities as filled contour plots using a graphic presentation software that constructs pseudo-colored isodensity maps on a scale with 38 different steps (each of 125) ranging from 0 to 4,750 cells/mm2. The plots should demonstrate the topological distribution of RGC labeled FG+ and Brn3a+.

NOTE: This upper limit was chosen on the basis of earlier studies that showed mean upper densities around this value14.

**REPRESENTATIVE RESULTS:**

DBA2/J mouse glaucomatous presents features of the human disease

*Intraocular pressure measurements*

The elevation of IOP is one of the hallmarks of most types of glaucoma. The measurements of mice IOP permit observation of the gradual increase in this physiological parameter, which starts to vary at 9 months and peaks at 12. Compared to the constant IOP of the control C57BL/6J strain, the glaucomatous mice demonstrate an increase. The maximal increase in IOP in the glaucoma mice (at 12 months) was 70%, compared either with the glaucoma strain before the onset of the pathology or with the control strain (Figure 1).

*Retinal Ganglion Cell Death*

Blindness is also another consequence of the glaucomatous pathology, and this is due to ganglion cell death. In this sense, the use of both OHSt retrograde tracing and Brn3a immunolabeling on the same retina allows for morphofunctional studies. While OHSt labels RGCs capable of retrograde axonal transport of the tracer from their target region in the brain towards their cell somata in the retina, Brn3a labels all surviving RGCs, regardless of their axonal transport capabilities14. Furthermore, the changes in the distribution of retrograde labeled (OHSt+) or immunolabeled (Brn3a+) RGCs can be examined and compared in detail by constructing contour plot isodensity maps for the retinas of each control or injured mouse (Figure 2).

*Electroretinogram responses*

To confirm the lack of functionality of mice retinas when glaucoma is developed, we performed STR recordings to assess the possible loss of function in the inner retina. ERG measurements are a useful tool to evaluate functional retinal changes in this animal model. At 12 months, compared with younger mice, glaucomatous mice showed significant reductions of electrical responses. However, there were no significant differences in ERG responses at any of the different ages studied in the control mice (Figure 3).

**FIGURE LEGENDS:**

**Figure 1.Intraocular pressure (IOP) measurements in the C57BL/6J and DBA/2J animals as a function of age.** IOP in C57BL/6J and DBA/2J animals was measured at 3, 9, and 15 months of age (white and black circles). Each circle corresponds to a mean ± SD of different animal measurements (n=8).

**Figure 2.** **Electroretinogram responses from control C57BL/6J or DBA/2J glaucomatous mice as a function of age.** Superposed ERG trace responses from C57BL/6J (A) and DBA/2J (B) mice recorded in response to light intensity (indicated to the left of the recorded traces in log cd·s·m-2). Recordings obtained from animals of 3 (green traces), 6 (blue traces), 9 (purple traces), and 12 (red traces) months of age are shown. A significant reduction in the ERG amplitude response was observed for the DBA mice. Averaged amplitudes of the positive waves (pSTR, circles) and negative waves (nSTR, triangles) measured from the ERG flash response as a function of stimulus light intensity recorded from C57BL/6J (open symbols) and DBA/2J (closed symbols) mice at 6 (C) and 12 (D) months of age. Plot data correspond to mean values ± SD (n=8). A significant reduction of STR amplitudes in DBA/2J between 12 and 15 months is observed for pSTR amplitudes (p <0.0001).

**Figure 3.** **Retinal distribution of surviving RGCs identified by Brn3a immunohistochemistry on 15-month-old DBA mice.** (A) Filled contour plots showing densities of Brn3a+ RGCs in whole-mounts. Note the intensely-labeled RGCs distributed throughout the entire retina, with the typical high-density region along the naso-temporal streak in the superior retina of a representative animal without RGCs loss (6 months of age). (B) Gradual absence of labeled RGCs preferentially in the superior retina as the pathology develops, adopting the form of focal as well as diffuse loss, as evidenced by the cooler colors of the isodensity maps (12 months of age). (C) Representative animal with severe damage showing labeled RGCs restricted to a wedge located between the 3 and 4 o’clock positions (15 months of age). Maps are represented as filled contour plots generated by assigning to each of the 36 subdivisions of each individual frame a color code according to its RGC density value within a 38-step color scale range from 0 (purple) to 4,750 (red) or higher (Brn3a+ RGCs/mm2). For all retinas, the superior pole is directed to the 12 o’clock orientation. Scale bar = 1 mm.

**DISCUSSION:**

In the DBA/2J mouse, the glaucomatous pathology can be monitored with at least three parameters: IOP, retinal ganglion cell loss, and retinal dysfunction37. This is a clear advantage compared to several models in which it is possible to study either changes in IOP, modifications in the retina, or changes in the electrophysiology, but never simultaneously. This implies that, depending on the particular interest, one or the other glaucoma model must be chosen38-40. The DBA/2J might not be the best model, but it depicts changes that mimic in many ways human glaucoma pathology and therefore opens the possibility for the investigation of this pathology from different perspectives.

As an added value, the present animal model will be useful to test new compounds, either for the reduction of IOP or for neuroprotection. The DBA/2J model has the advantage of presenting the common symptom, abnormal IOP. In this sense, it is of interest to use this model to test potential anti-glaucomatous drugs designed to reduce IOP. The moment of maximal IOP (12 month of age) is an optimal time point to test these molecules. The application of classical hypotensive drugs significantly reduces IOP, indicating that this model is suitable for this type of study.

Since it is possible to follow retinal degeneration as a progressive event from nine months of age onwards, neuroprotective agents could also be explored. As controls, we may use animals treated with vehicle, while experimental groups may be treated with the desired compound. Moreover, ERG recordings from such animal groups will allow investigations of the putative neuroprotective effects of new substances. It will also be possible to establish optimal treatment timeframes in order to get the best final results. It is important to notice that the only problem of this approach is the time necessary for the animal to develop the pathology. It is necessary to wait a minimum of 9 months to start to see the changes in IOP and the retinal impairment.

These two treatment development strategies are possible due to the gradual progression of the pathology, which permits research into the establishment of the most suitable treatment start point. Although the DBA/2J mouse presents several limitations, it offers several advantages above the other existing animal models of glaucoma.

**DISCLOSURES:**

The authors have nothing to disclose.

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