ISSN (P) : 2589-9120 / (O) : 2589-9139
PubMed-National Library of Medicine - ID: 101773527

International Journal of Medical Science and Applied Research (IJMSAR)

Available Online at: https://www.ijmsar.com

Volume – 5, Issue – 2, April – 2022, Page No. : 23 – 33

Preclinical Models for Glaucoma Drug Discovery

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Citation of this Article: Dr. Prakash N Patil, Dr. Yashashri Shetty, "Preclinical Models for Glaucoma Drug Discovery ,'' IJMSAR – April – 2022, Vol. – 5, Issue - 2, Page No. 23-33.

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Type of Publication: A Review Paper

Conflicts of Interest: Nil

Abstract

Glaucomais defined as aneurodegenerative disease characterized by loss of retinal ganglion cell (RGC) and optic nerve atrophy. Increased intraocular pressure (IOP) is a major risk factor for onset and progression. Aselevated IOP is the only modifiable risk factor, glaucoma models would contain RGC and optic nerve damage set off by ocular hypertension. In vivo models of glaucoma have significantly contributed to the understanding the pathological molecular mechanisms involve in glaucoma, and they have also facilitated the development of new pharmacological interventions. While glaucomaanimal models have provided important information about glaucoma, there is still no ideal model for studying glaucoma because of its complexity. There is a demand for in vitro models that can reduce& replace the need for animal experiments. Many in vitro models have come to light as a great opportunity in theglaucoma researchfield, helping to explain the mechanisms involved in disease progression. In this paper, we present *in vitro*&*in vivo* glaucoma models that have been developed for research in different types of glaucoma along with variables used and limitations.

Keywords

Retinal ganglion cell, Increased intraocular pressure, Glaucoma

Key Messages

Pathophysiologic mechanisms causing the Glaucoma are not completely understood. Animal models of glaucoma have greatly contributed to the understanding of the molecular mechanisms to its pathology but still no ideal model for understanding glaucoma due to its complexity so no doubt that more studies are needed in this important research field of

Glaucoma which will allow the development of new relevant models to study RGC neurodegeneration and neuroprotection in the context of Glaucoma.In this paper, we present *in vitro* & *in vivo* glaucoma models that have been developed for research in different types of glaucoma along with variables used and limitations.

Introduction

Glaucoma is a group of disorders that all have in common specific patterns of optic nerve damage irrespective of the intraocular pressure (IOP). It is ophthalmic neuropathy characterized by Progressive loss of Retinal Ganglion Cells & Concomitant loss in the field of vision. Glaucoma is second mostleading cause of irreversible blindness [1], affecting over 60 million people globally. Its prevalence has been projected to increase by almost 75%by the year 2040. Optic nerve acquires a characteristic loss of the neuro-retinal rim, frequently referred to as "cupping" which results in progressive loss of vision because of increase in IOP. Studies shows each additional mmHg IOP increase 11% risk of glaucoma progression [2]. Neuronal growth factors, peroxy-nitrile toxicity, immune mediated nerve damage & oxidative stress factor are contributing factors but the most important factor responsible for Glaucoma is neurodegeneration in the form of Retinal Ganglion Cell Injury [3]. Current pharmacological therapy (e.g. PGF2α analogues, β receptor antagonists, α 2 receptor agonist, Carbonic anhydrase inhibitor) targets only decrease in IOP either increase in Uveoscleral outflow or decrease in Aqueous humor production. With this current line treatment even with good compliance 15%-20%of patients become blind in at least one eye in 15 to 20 years of follow-up[4]& data suggests Laser trabeculoplastywhich is considered to be gold standard for management of chronic open angle glaucoma have a 50% failure rate after 2-5 years[5]. So there is an unmet need in drug therapies for glaucoma.

In drug development Laboratory (in vitro) & animal (in situ or in vivo) studies are conducted to show the pharmacological activity (efficacy) of the compound against Glaucoma and to determine the compound's toxicological activity (safety)&after completing sufficient preclinical testing, a company files an IND(Investigational new drug) which has to pass through Phase I-III & finally NDA(New Drug Application) through which drug sponsors propose that the FDA approve a new drug formarketing and sale i.e. Phase IV.Models of Glaucoma have greatly contributed to the understanding of the molecular mechanisms of this pathology but still there is no ideal model which could study the complexity of glaucoma. Development of new relevant modelsto study RGC neurodegeneration and neuroprotection in the context of glaucoma is needed.

Text

In Vitro Models

1. The EHP Model

Elevated hydrostatic pressure (EHP) model considered to be the gold standardmodel for ocular hypertension in in vitro systems. many systems have been developed to determine the responses of tissue or cell to elevated pressure. In themajority of glaucomatous patients, the IOP values vary from 20 to 35 mm Hg. To understand the RGC loss in glaucoma, in vitromodels should recapitulate the effects of elevated pressure in relatively short time periods. In fact, most of the data mimicking chronic IOP elevation use pressures ranging from 30 to 100 mm Hg above the atmospheric pressure for 10 min to 72 h. Many systems designed to attain raised pressure are based on pressurized chambers built in polymethyl methacrylate and connected to air/CO_2source with a pressure

regulator, and they are kept in a temperature-controlled environment. These systems provide a constant hydrostatic pressure within ±1 mm Hg and allow stable control of the pressure influx to the chamber, while control cells are usually kept at atmospheric pressure (760 mm Hg) in standard cell incubators or in some cases exposed to 15 mm Hg to mimic normotensive conditions. Cell lines (RGC-5, B35, and PC12), primary cultures of RGC and astrocytes, Muller cell cultures, or microglial cell cultures, and more complex preparations like organotypic retinal cultures [6]and eye cuppreparations [7]have been exposed to EHP. In addition, human retinal cells, human organotypic retinal cultures [8], and human optic nerve head (ONH) astrocytes [9]have been exposed to EHP.

Chambers for Elevation of Pressure

Pressurized Chamber

The most commonly used model of a pressurized chamber injects a mixture of humidified gas (95% air/5% CO_2) through a pressure regulator into a polymethyl methacrylatebox. This chamber is kept at 37 ° C in a standard incubator to allow temperature and humidity stabilization. The pressure inside the equipment is constantly monitored using a diaphragm-driven dial pressure gauge and it is kept stable within ±1 mm Hg, from 0 to 200 mm Hg. Studies using this type of pressure chamber have demonstrated that exposure ofneuronal cell lines (B35 and PC12) to 100 mm Hg for 2 h triggers apoptosis, establishing a direct relationship between pressure and neuronal loss [10]. In addition, exposure of PC12 cells to 15 or 70 mmHg for 24 h and of RGC- 5 cells to 30 mm Hg for 72 h also induces apoptosis, oxidativestress, mitochondrial changes, and a reduction in cellular ATP levels, supporting the role of mitochondrial oxidative injury in early glaucomatous damage [11-12].

In one study authors reported that exposure of a Muller cell line or organotypic retinal cell cultures obtained from mice and primates to different levels of pressure (15, 33and 46 mm Hg) for 24 or 72 h doesnot alter the expression of the complement genes but promotes an increase in the expression of glial fibrillary acidic protein (GFAP) [13], contrary to what was described in human glaucomatous samples [14]. These apparently contradictory results raise the question of whether in vitro models are suitable for the study of glaucoma, but they may also be explained by a lack of activated T cells [15] that may have a role in the human condition.

A study reported microglia activation, RGC death and up regulation of adenosine A 2A receptors in retinal microglial cell cultures following exposure to 70 mm Hg for 4 or 24 h [16].Using this model, the authors unravelled a possiblemechano sensitive role for retinal microglia which become reactive and promote the inflammatory response following an EHP challenge. Interestingly, neutralization of the actions of the proinflammatory cytokines tumour necrosis factor (TNF) and IL-1β prevents RGC death, pointing to neuroinflammation as a key mediator of the neurodegenerative process triggered by EHP [17].

Hydraulic Pressurizing Chamber

A hydraulic pressurizing chamber is composed of 2 chambers, a gas filling system, a hydraulic pump, and a hydraulic cylinder. The pressure cylinder is made of steel, while the non-pressurized chamber is made in poly vinyl chloride plastic. The pressure inside the chamber is obtainedusing a hydraulic system where the oil flow generated by the hydraulic pump is conducted to the hydraulic cylinder underneath the pressure chamber. Elevated pressure is obtained via compression of the gas phase inside the chamber. The pressure is regulated by a pressure valve, which transfers the

necessary force to the chamber filled with 95% air/5% CO_2 via a computer-controlled system. This system is kept at 37 ° C in a standard incubator and the pressure is constantly monitored. In this case, the control cultures are kept in the reference non-pressurized chamber.

This device has been used to challenge human ONH astrocytes at 60 mm Hg for 6 or 48 h, inducing cell migration [18]and causing alterations in cell morphology and the distribution of GFAP, increasing the levels of neural cell adhesion molecule and small heat shock protein 27 [19-20]. These proteins are involved in cytoskeleton alterations and cellular migrationof reactive astrocytes, and they are increased in astrocytes of the ONH of patients with glaucoma [19-20].

In vitro experiments may also provide important data on the impact of elevated pressure in cells obtained from human populations with different susceptibilities to glaucoma. The prevalence of glaucoma is higher in Black Americans of African American ancestry than in Caucasian American populations of European ancestry [21]. Exposure of ONH astrocytes from Caucasian and African American donors to 60 mm Hg for 24 to 96 h increases the expression of the myosin light chain kinase isoforms (MYLK-130 and MYLK-210)[22]. However, transforming growth factor-β 2 (TGFβ 2) was found to be upregulated only in ONH astrocytes of Caucasian donors [22]. Thus, TGFβ 2 upregulation triggered by EHP in ONH astrocytes of Caucasian donors suggests that it may be an important indicator of reactive astrocytes in vitro and glaucomatous alterations in the ONH. This group of results demonstrates that different susceptibilities to the development of glaucoma may be further studied in vitro, allowing the identification of potential molecular targets for different human populations.

Pressurized Live-Cell Imaging Chamber

The pressurized live-cell imaging chamber has been installed on the stage of an Olympus IX71 inverted optical microscope. In order to increase the pressure inside the chamber to 100 mm Hg, a mixture of pre-humidified and heated 95% air/5% CO_2 is injected through a pressure regulator at 37 °C under continuous perfusion. Exposure of RGC-5 cells to EHP (100 mm Hg for 20 h) induces apoptosis and morphological changes mediated by early alterations in Ca 2+ dynamics and caspase-3/7 activation [23]. Unfortunately, one major disadvantage of this experimental setup is that the control and experimental procedures are not performed at the same time, which may lead to an increased variability in cell response.

Pressure variation Chamber

There is mounting evidence that IOP fluctuations playan important role in the development and progression ofglaucoma [24]. A pressurized chamber was designed to mimic IOPoscillations by performing cycles of 10 and 100 mmHgeach minute for 1 h. The chamber is connected to a massflow controller which injects a mixture of 95% air/5%CO_2 that simultaneously regulates the internal pressureand the gas flow.

Exposure of primary rat RGC culturesto either fluctuating pressures from 0 to 30 or 60 mmHgor a constant pressure of 30 or 90 mmHg for 72 h doesnot elicit cell apoptosis but does increase the susceptibility of RGC to glutamate excitoxicity, which has been considered to play an important role in glaucomatous degeneration [25]. One major point that must be taken into consideration is that pressure oscillations in the human eyeare dependent on the circadian cycle, which is difficult to model in vitro [26].

2. Pressurized Cell Culture Flasks

In this setup, T75 culture flasks are adapted with a pressure gauge and a syringe to pump the air mixture of 95% air/5% CO_2, while control culture flasks are maintained in a standard cell incubator [27-28]. Using this system, exposure of primary Muller cell cultures to 40 mm Hg for 24 h increased the expression of inwardly rectifying potassium channels, glutamate aspartate transporter, and glutamine synthetase, indicating that Muller cells are susceptible to EHP [27-28].However, incubation of cells with drugs or replacement of the culture medium requires the system to be disconnected from the air source, introducing handling bias.

3. Centrifugal Force Loading Model

Another model with the purpose of reproducing a state that closely mimics the pressurized state was developed by applying centrifugal force. The device comprises a rotating vessel installed within an incubator, a power supply unit, a control unit, and a cooling motor. The rotor spinsat 1–30 rotations per minute and the cells experience a centrifugal force equivalent to 16, 28or 48 mm Hg [29]. Exposure of primary retinal glial cell cultures to a centrifugal force equivalent to 16,28 or 48 mm Hg for 48 h did not altercell survival. Interestingly, the exposure of cocultures of RGC and glial cells promotes viability of RGC, indicating that retinal glial cells exhibit protective effects on RGC subjected to centrifugal force loading [29].

4. Stretch-Based Models

In a glaucomatous eye, elevated IOP induces compression, stretching, and rearrangement of the cribriformplates in the ONH, resulting in cupping of the optic disc. Lamina cribrosa cells in the ONH arecontinuously exposed to mechanical strain. Models inducing mechanical stretch have been also developed tostudy changes occurring in glaucoma. These modelsuse commercially available straining systems allowing asustained increase in strain or variable pulses [30],whereas control cells are kept in static conditions. When cultures of human lamina cribrosa cells exposedto a mechanical strain of 15% stretch at 1 Hz for 24 hchanges genes expression related to cell proliferation, growth factor activity and signal transduction [30].Furthermore, stretch changes the expression of genes thatregulate the extracellular matrix in these cells, which isprevented by the blockade of calcium channels, highlighting the role of calcium influx in matrix remodelling inglaucoma [31]. These datastipulate that stretch-based method may alsobe relevant to the study of alterations in glaucoma, i.e. tobetter understand cell-to-cell communication and structural alterations.

Advantages of In vitro models [32]

- Stable control of the temperature and pressure influx inside thechamber.
- Cell lines or primary cultures allowing controlled cellular interactions
- Requires Less Periodranging from 10 min to 120 h.
- Constant or fluctuating pressure, different time points and pressure intensities because of different experimental settings.
- Easier to replicate than animal studies, use of cell lines highly decreases variability.
- Treatment is given to cell lines or primary cultures which reduce animal distress.

Disadvantages of In Vitro Models [32]

- In experimental conditions Control cultures are often not in the same cell incubator.
- Pressure application of 30 to 100 mm Hg above the normal atmospheric pressure.

In Vivo Models

Primary open angle glaucoma (POAG) is the most common form of glaucoma characterized by elevated IOP and acquired RGCs loss and ONH atrophy. Spontaneous and induced animal models that have been used to study POAG, and provided valuable details about the disease are described below.

5. Monkeys

Glaucoma in monkeys was first describedwhen a group of rhesus monkeys screened for diseases in the posterior segment of the eye were found to haveboth low and high (>or =22 mmHg) tension POAG [33]. Rhesus monkey POAG was found to be of maternalinheritance IN about 40% demonstratingincreased IOP. Affected animals exhibit a RGCs loss, optic nerve(ON) excavation, and electrophysiological findingof retinal peripheral field damage.

To study POAG many experimental monkey models have also been developed. Gaasterland and Kupfer developed an experimental monkey model using argon laser photocoagulation [34]. They used a modified Koeppe direct goniolens to laser the entire trabecular meshwork circumference which lead to IOPelevationin about 70%ofthe animals. After the 4th treatment IOP range was 24-50 mmHgand remained raised for 25 days. Histopathology from eyes with elevated IOP and ON cupping showed RGCs loss and nerve fibres thinning compared with histopathology of untreated controls suggesting that glaucoma was attained. Many studies after that used the monkey model to show the functional and anatomic changes that occur within the eye and ON in an effort to recognize the reasons that result in IOP elevation [35-36]. Other experimental monkey models of chronic IOP elevation were developed by using latex microspheres [37] and autologous fixed RBC [38].

The similar phylogeny and maximum homology of the monkey with humans makes it an excellent model for glaucoma. They have retinal and ON anatomy that is almost identical to humans. Sadly, monkeys are very costly, limited availability, and they are difficult to handle. Experiments using monkeys require highly skilled teams and special housing facilities, making them beyond the reach of many research laboratories.

6. Mice

A mouse strain with Tyr423Hismyocilin point mutation matching to the human MYOCTyr437His mutation was developed to study POAG [39].Myocilin is one of the causative genes of POAG in humans[40] and has been extensively studied. At 18 months of age,the myocilin model demonstrated ~20% RGCs loss in the peripheral retina, axonal degeneration in the ON, trabecular meshwork endothelial cells detachment, and moderate and persistent elevation of IOP(2 mmHg higher than normal) [41].

Another transgenic mouse strain with a targeted mutation in the gene for the collagen type I (α1 subunit) was developed to study POAG. This model demonstrated open angles, progressive ON axonal loss, and gradual elevation of IOP indicating an association between IOP regulation and fibrillar collagen turnover [42-43].

There are several superiorities of using mice in glaucoma research. These comprise the high degree of conservation between mice and human genomes, enabling genetic manipulation by altering the mouse genome, and the ability to breed the animals as desired. they are economical and easy to house and handle, their eyes are easy to get, and larger sample size. The

drawbacks of the mouse model are the absence of the lamina cribrosa in the ON, very small globe size which makes it difficult to access clinically, and limited availability of specific models.

7. Rats

A rat model of glaucoma, induced by topically dexamethasone application, was also developed to study the myocilinexpression. Although IOP was increased after 2weeks of induction, the protein and mRNA levels of myocilinin the trabecular meshwork and around Schlemm's canal in the induced eyes were not different from those of the controls indicating thatmyocilin may not be directly linked to ocular hypertension[44].

Similar to mice, rats have many advantages. Rat shares identical anatomical and developmental characteristics of the anterior chamber, precisely in the aqueous out flow pathway, with the human. Therefore, results obtained from the ratare expected to similar changes that occur in the human. Furthermore, there is reasonable IOP rise as retinal and ON changes are similar to those seen in humans. Also, IOP reduction in response to glaucoma medications has been described but the medication effects were not all identical to those observed in humans [45]. In addition, rats are easier to maintain in the laboratory and similar to mice they enable genetic manipulation and can be used in large numbers.

8. Rabbits

Administration of glucocorticoids can lead to the development of ocular hypertension and POAG through a reduction in aqueousoutflow [46]. Models using steroid-induced ocular hypertension have been also developed. In rabbits, betamethasone injection sub-conjunctively or α-chymotrypsin injection into the posterior chamber also resulted in raised IOP that lasted for 7 weeks [47]. IOP response consistency as well as robustness and the low cost of maintaining the rabbits developed using steroids compared to primates are all advantages of this model. But the prolonged topical corticosteroid treatment required to achieve glaucoma can cause significant adverse effects such as cataracts and corneal ulcers are major disadvantages.

Conclusion

In vivo models utilized in glaucoma have provided valuable information about certain aspects of the disease process but the search for models that address knowledge gaps in specific forms of glaucoma will continue. The validity of each of these models depends upon the degree of similarity to the human condition as well as considerations of the model being economical and practical. Since the glaucoma mechanisms vary among animal models, data acquired from a particular model should not be generalized and should be interpreted within the context of that model. The animal model used should be selected based on the needs of experiment and the hypothesis being tested.

In vitro models will not at all substitute animal studies, but they are dominant tools in preclinical studies in the glaucoma field. Thoughless complex than animal models, in vitro models of EHP offer many advantages like controlled experimental conditions, clarifying individual cell responses to stress and allowing preliminary targeting of a specific cell type or pathway involved in the glaucoma. There is no doubt that more studies are required in this research field, which will allow the development of newmodels to study RGC neurodegeneration and neuroprotection in the context of glaucoma.

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