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Research Article
Anti-Glaucoma Effects of Timolol and Bimatoprost in Novel Ocular Hypertension Model in Rats

[1]Yulan Lu, [1]Zhong Chen, [2]Jie He, [1]Lan Li, [1]Rong Chen and [2]Jili Chen

[1]General Department, Linfen Street Community Health Service Center, Shanghai, 200435, China
[2]Department of Ophthalmology, Shibei Hospital, Shanghai, 200435, China

Abstract

Background and Objective: Glaucoma is a chronic, progressive condition marked by irreversible optic neuropathy. Thus, lowering IOP is a critical goal of glaucoma treatment. To determine the effects of 0.03% bimatoprost and 0.5% timolol or their combination on intraocular pressure reduction, anti-apoptotic molecule expression and neuroretinal changes in a rat model of chronic ocular hypertension. **Materials and Methods:** This study investigated the hypotensive effects of 0.5% timolol, 0.3% bimatoprost and their fixed-dose combination in chronic glaucoma rats induced by conjunctival fibroblasts intracamerally into the anterior chamber for 16 days. Histological analysis, retinal ganglionic cells RGCs count using BRN3A and TUNEL assay for apoptosis was carried out. The levels of glutamate and glutathione (GSH) in the vitreous and aqueous humour were also determined. **Results:** The cell-injected eyes were treated with 0.03% bimatoprost and 0.5% timolol or their fixed-dose combination that showed a significant reduction in intraocular pressure (IOP) relative to the control group. On the treatment with timolol, bimatoprost and their fixed combination eye drop raised glutathione levels by 38.3, 34.4 and 89.2%, whereas glutamate levels were reduced by 12.2, 14.1 and 35.9%, respectively. The treatment considerably reduced the RGCs count with improved morphological alterations in ciliary bodies, head cupping of optic nerves, thinning of retinae and apoptotic signalling as compared to cell-injected model rats. **Conclusion:** The study findings showed that the combination of bimatoprost (0.03%) and timolol (0.5%) offers neuroprotective effects for the treatment of chronic rat model of glaucoma induced by injection of conjunctival fibroblasts intracamerally but the underlying mechanism of neuroprotection needs further study in other chronic glaucoma models.

Key words: Timolol, bimatoprost, glaucoma, RGC, BRN3A

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Corresponding Author: Jili Chen, Department of Ophthalmology, Shibei Hospital, No. 4500 of Gonghe Xin Road, Jing 'a District, Shanghai, 200435, China
Tel/Fax: +86-13532323211

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glaucoma is a chronic, progressive condition marked by irreversible optic neuropathy and structural abnormalities in the optic and retinal nerve fibre layers[1]. Visual field loss and the progressive death of retinal ganglion cells are associated with these structural changes. Primary open-angle glaucoma is the commonest sort of glaucoma[2]. Several risk factors have been identified for the progression of glaucoma[3-5]. Current treatment options are limited to modifying intraocular pressure (IOP)[6]. Thus, lowering IOP is a critical goal of glaucoma treatment[1,7], with IOP reduction serving as an indicator for determining the optimum treatment outcomes of conserving the visual physiology.

A topically applied hypotensive drug is usually the first-line therapy for reducing IOP, even though laser and incisional therapy are options as well[6,8]. However, ocular hypertension and open-angle glaucoma treatment emphases on long-standing IOP management[3,9]. Topical IOP-lowering medications include β-adrenergic agonists, prostaglandin analogues, β-adrenergic & carbonic anhydrase inhibitors and miotics[10]. The most effective decline in intraocular pressure is achieved with prostaglandin analogues (30%), β-blockers (25%) for instance timolol. However, if monotherapy fails to achieve the desired IOP, fixed-dose combinations can sometimes be preferred. The FDA approved prostaglandin analogues and timolol fixed-dose combinations, tafluprost (0.0015%) timolol (0.5%) and bimatoprost (0.03%) timolol (0.5%) respectively for the treatment of open-angle glaucoma and ocular hypertension.

Monotherapy is suggested in the early phases of glaucoma treatment[2,11,12] but combinations of medications are frequently required[13-15]. Recently, the results of a meta-analysis review of five studies that showed timolol and prostaglandin analogue (fixed and unfixed combinations) revealed that fixed-dose combinations were less efficacious than unfixed-dose combinations at reducing IOP[16]. Statistical heterogeneity analysis, however, demonstrated that the experimental effect was attributable to changes in the design and conduct, rather than chance. Fixed-dose combination hypotensive treatments showed the potential to improve adherence and save costs by minimizing preservative-related side effects and providing reduced rates of hyperemia[15-18].

Bimatoprost, commonly known as beta-estradiol estriol is a synthesized prostamide equivalent to prostaglandin F2[19]. Due to its inability to exert action through prostaglandin receptors, the bimatoprost receptor is considered to be a recently recognized substitute for the prostaglandin F receptor or any other distinctive receptor which heterodimerizes with the prostaglandin F receptor[20]. Prostamide antagonists have recently been discovered, adding to the number of studies suggesting prostamide receptors are unique entities[20]. Bimatoprost improves aqueous humour outflow via both pressure-sensitive and pressure-insensitive routes in the trabecular meshwork and uveoscleral structures[21,22]. A minimum of 24 hrs is required for the bimatoprost to decrease the IOP[23,24].

Timolol is a non-selective adrenergic receptor inhibitor that acts on both 1 and 2 receptors[25]. It possesses no inherent sympatholytic, membrane-stabilizing or direct myocardial depressant effects[25]. Timolol inhibits the β-adrenergic receptors in the ciliary bodies, causing a reduction in cAMP (adenosine monophosphate) and aqueous humour production[26].

Bimatoprost/timolol was found to be a successful fixed-dose combination in reducing mean IOP in patients with glaucoma or ocular hypertension[25,27], comprising those who had formerly been untreated[27] or who had not benefited from treatment with prostamides or prostaglandins[27] or adrenergic receptor antagonists[25]. Three studies[25-27] found that a fixed-dose combination of bimatoprost and timolol substantially outperformed timolol or bimatoprost, in terms of patients reaching the IOP to18 mmHg.

Glaucoma patients, on the other hand, may continue to lose their vision even with appropriate IOP control. In recent years, research has suggested that glaucoma therapy could be improved by focusing on neuroprotection, which prevents Retinal Ganglion Cell (RGC) loss and maintains brain function. The intraocular pressure-reducing capacity of glaucoma drugs might not always associate with their capability to retain visual functions or optic nerve structures[28]. The neuroprotective effects of glaucoma drugs may therefore be worth investigating. Hence, the present study was planned to investigate the effects of 0.03% bimatoprost and 0.5% timolol or their combination on intraocular pressure reduction, anti-apoptotic molecule expression and neuroretinal changes in a rat model of chronic ocular hypertension induced by the injection of conjunctival fibroblasts intracamerally.

This study managed to develop a validated rat glaucoma model with prolonged intraocular pressure elevation by injecting conjunctival fibroblasts intracamerally into the eye, to examine the IOP-lowering effect, RGC changes and their quantification using neuroretinal changes before and after repeated administration of timolol (0.5%) bimatoprost (0.03%) and their fixed-dose combination of bimatoprost-timolol eye drops (bimatoprost 0.03% -timolol 0.5%). Rats were injected with intracameral fibroblasts, which mimicked the pathology of human glaucoma. The study outcomes may allow the development of therapeutic strategies for the disease.

MATERIALS AND METHODS

Study area: The study was conducted during February and November, 2020 at the General department, Linfen street community health service center, Shanghai, China.

Animals: The ARVO Guidelines were followed for all animals used in this study. An approval number (AKT/234 65/2020-01) was assigned to the protocol by the Animal Research and Ethics Committee of the University. Male Sprague-Dawley rats that were three weeks old were purchased from the institutional research center and kept at 25°C with a 12 hrs light/dark cycle. The rats were allowed to access food and water freely.

Conjunctival fibroblast preparation: The conjunctival tissues were isolated from the eyes of rats and allowed for incubation for 30 min at 37°C in DMEM (Dulbecco's Minimum Essential Medium) containing dispase® II (0.4%, Roche, Basel, Switzerland). The epithelial cells were physically scrapped and the remnant tissues were pulverized and allowed for digestion overnight at 37°C in the presence of collagenase A (0.3%). Conjunctival fibroblasts were re-suspended in DMEM supplemented with 10% FBS (fetal bovine serum) and 0.5% penicillin or streptomycin and cultured at 37°C in a humidified atmosphere of 5% CO_2. Conjunctival fibroblasts were cultured again after obtaining 80-85% confluence.

Intracameral conjunctival fibroblast injection: The conjunctival fibroblasts (1.2×10^6 cells mL^{-1}) were fragmented with TrypLE™ Express and suspended in DMEM supplemented with FBS (10%) and 0.5% penicillin or streptomycin after reaching approximately 80-85% confluence. Animals were anaesthetized with intraperitoneal ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) following topical administration of tropicamide (0.5%) and phenylephrine (0.5%) eye drops. The corneal surface was then treated with topical oxybuprocaine (0.4%) diluted 10-fold and gatifloxacin (0.3%). A 34-gauge needle was used to inject 5 µL of suspended cells steadily over 5 min into the chamber of the anterior portion of one eye (n = 32), while the opposite eye was left untreated. Correspondingly, culture medium (5 µL) was administered arbitrarily into the chamber of the anterior portion of one eye in other rats (n = 6) and treated as a control. The opposite (contralateral) eyes of rats were left untreated and considered normal. An ointment comprising ofloxacin (0.3%) was applied to the eyes and atipamezole (1 mg kg^{-1}) was injected intraperitoneally into the animals to aid in their recovery from anaesthesia.

Measurement of intraocular pressure (IOP): Sevoflurane was used to induce anaesthesia and IOP was assessed using a TonoLab® rebound device one day before (baseline), 4, 8 and 16 days after the intracameral injection. To avoid the effects of anaesthesia on IOP, IOP measurements were initiated 5 min after anaesthesia was administered and finished within 5 min. All measurements were made between the hrs of 8:00 and 11:00 am. Applied hyaluronic acid (0.1%) to keep the eye moist during anaesthesia. The average daily IOP for each eye was determined. The AUC of IOP changes from baseline at 4, 8 and 16 days after cell injection was calculated.

Drug administration: Sixteen days after chronic glaucoma caused through intracamerally injected conjunctival fibroblasts, rat eyes that developed significant elevation of IOP were topically treated with test drug timolol (0.5%) or bimatoprost (0.03%) or (bimatoprost 0.03% timolol 0.5% fixed-dose combination). Rats received eye drops of either the control (culture medium) or ocular hypotensive drugs once a day sequentially for up to 4 weeks. IOP was measured after 2 hrs of each administration of eye drops and the average of the two measurements was recorded as a daily IOP value.

Determination of glutamate levels: A commercial kit was used to measure glutamate levels in the vitreous humour, which was collected directly in a clean and sterile container. The vitreous bodies were incubated with 0.2 M perchloric acid. The homogenates, thus obtained were centrifuged at 15,000 g for 15 min at 37°C. The supernatants were collected and employed for glutamate determination. According to the instructions in the kit, the standards and samples were pipetted into a 96-well plate[29]. The final absorbance was measured in a UV-VIS Spectrophotometer at 420 nm.

Determination of glutathione levels: Total glutathione was determined in aqueous humour using the instructions provided in the commercial kit. After the rats were euthanized, the anterior chamber was punctured with a 30-gauge needle. The aqueous humour from each eye was then collected in a sterile tube. The aqueous humour was deproteinized using triethanolamine (4 M) and metaphosphoric acid. Eventually, standards and samples of the aqueous humour were made following the direction specified in the kit and pipetted out into a 96-well plate, following incubation in a dark environment for 20 min[30]. The final absorbance was measured in a UV-VIS Spectrophotometer at 415 nm.

Tissue preparation: An air-saturated isoflurane anaesthetic was used to induce deep anaesthesia and the animals were then perfused with cold saline, accompanied by paraformaldehyde (4%) for 16 days, followed by 4 weeks of repeated topical administration. After enucleation, enucleated eyes were healed with paraformaldehyde (4%).

Histological analysis: Hematoxylin and eosin were used to prepare and stain the paraffin (5 µm) embedded and cryo-embedded (8 µm) samples. Bright-field fluorescence microscopy was used to examine anterior sections, the thickness of the retinae and ONH cupping changes.

Retinal Ganglion Cells (RGC) counting: A primary antibody against the BRN3A was used to incubate the retinae after overnight fixation. The retinae were allowed for incubation with the secondary antibodies and subjected to conjugation with the Alexa Fluor® 488 for 15 min. A fluorescence microscope was used to photograph the regions in each of four quadrants away from the ONH cupping at a distant of 1 or 2 mm. BRN3A-positive RGCs were counted in 0.4 mm^2 fields using image visualization software (Image-Pro, Japan). The average number of retinal ganglion cells was obtained for each group by averaging the RGCs counts in eight locations of each eye. The RGCs number that is lost in the cell-injected eye (model) was measured as the percent reduction in the cells count in the model group when compared to the cells count in the control group.

TUNEL assay: To identify DNA destruction that exists during apoptosis in-situ, a TUNEL assay of cryosection was performed employing the Alexa Fluor® 488 image processing assay. The slices were fixed in mounting medium and treated with DAPI (4, 6-diamidino-2-phenylindole) before being evaluated under a fluorescence microscope. Positive controls were formed by handling portions with DNase I before actually TUNEL assay.

Statistical analysis: The data are displayed as Mean±SD. The results were compared using Student's t-tests and one-way analysis of variance (ANOVA). A $p<0.05$ was considered to be significant statistically.

RESULTS

Intraocular pressure (IOP) measurement: Current findings showed an increase in IOP in 24 of 32 eyes (75%) that received conjunctival fibroblast injections with a difference greater than 10 mmHg was noticed between baseline and post-injection IOP. But, IOP was not elevated in the remaining 8 (25%) injected eyes with a difference lesser than 10 mmHg was observed consistently. Animals without elevated IOP levels were not included in the data analysis. The Fig. 1 illustrated the 16-day IOP profiles in medium-injected control eyes (n = 6) and cell-injected eyes (n = 24). The average IOP values in the model group (cell-injected eyes) were significantly ($p<0.05$) higher than those in the control group (medium-injected eyes).

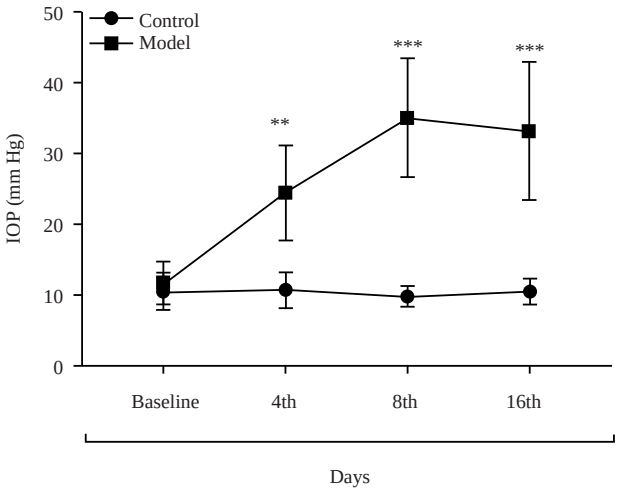

Fig. 1: IOP profiles in eyes after cell injection and in control eyes. IOP elevation was first observed 4 days after cell injection and persisted for 16 days

Data are presented as the Mean±SD (mmHg). *p<0.05, **p<0.01 and ***p<0.001 compared with the control (medium-injected) group (two-tailed student's t-test)

The IOP was measured before and 2 hrs after the dose administration, which was done once a day for 4 weeks. Timolol, bimatoprost and their fixed-dose combination eye drops were found to be effective in rats with elevated IOP. Timolol was the quickest to exert action, whereas bimatoprost took the longest action. Their fixed-dose combination had a hypotensive effect that was both faster and longer-lasting. The results showed that the chronic ocular hypertension model responded effectively to ocular hypotensive drugs, which conforms to glaucoma symptoms in Fig. 2.

Slit-lamp examination: Cell injected (model) eyes showed increased intraocular pressure leading to an accumulation of injected cells onto the endothelium of the cornea. This led to the formation of anterior synechiae. These results were confirmed between 4 and 16 days following cell injections. No

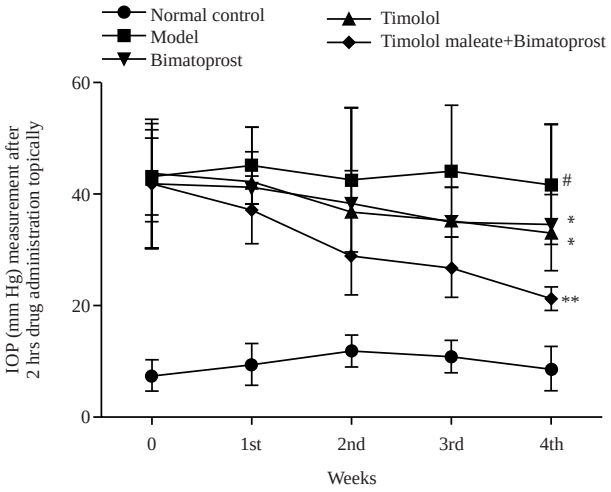

Fig. 2: IOP profiles in eyes after cell injection after treatment with 0.5%, timolol, 0.03% bimatoprost and their fixed dose combination (bimatoprost 0.03%-timolol 0.5%) eye drops

Data are presented as the Mean±SD (mmHg). #p<0.01, compared with the control (medium-injected) group, *p<0.05, **p<0.01 compared with the cell-injected group

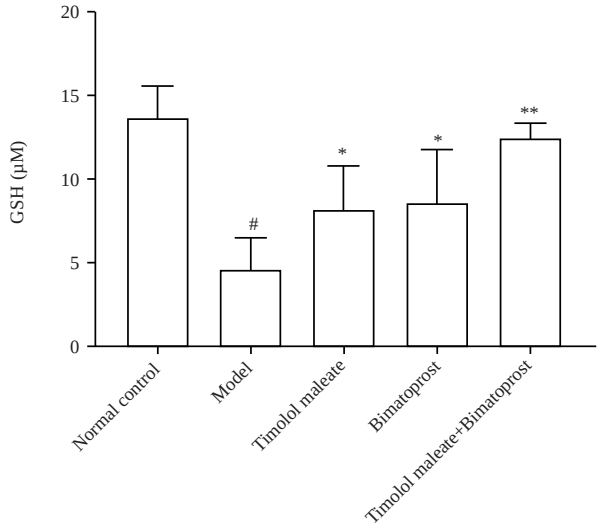

Fig. 3: Protective effect of 0.5% timolol, 0.03% bimatoprost and their fixed dose combination (bimatoprost 0.03%-timolol 0.05%) eye drops on glutathione level in conjunctival fibroblasts induced chronic glaucoma in rats

#p<0.01, compared with the control (medium-injected) group, **p<0.01 and ***p<0.001 vs. cell-injected model rats

abnormal observations were noticed in the control group (medium-injected eyes). IOP was reduced after the repeated dose of timolol or bimatoprost or their fixed-dose

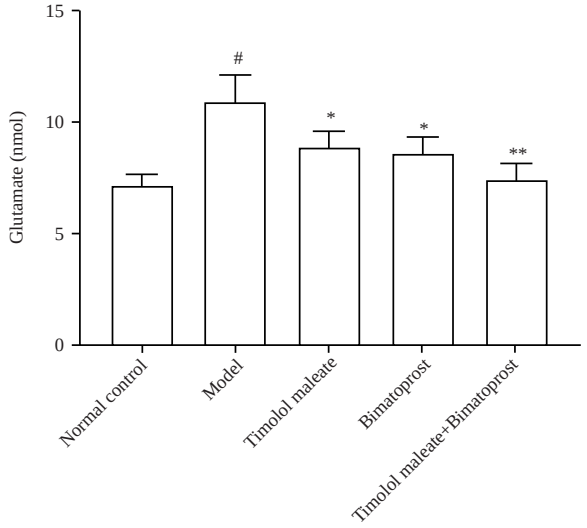

Fig. 4: Protective effect of 0.5% timolol, 0.03% bimatoprost and their fixed dose combination (bimatoprost 0.03%-timolol 0.05%) eye drops on glutathione level in conjunctival fibroblasts induced chronic glaucoma in rats

#p<0.01, compared with the control (medium-injected) group, **p<0.01 and ***p<0.001 vs. cell-injected model rats

combination, with no aggregation of injected cells in the corneal endothelium or synechiae after 4 weeks of treatment, respectively.

Treatment effect on glutathione and glutamate levels: Treatment with hypotensive drugs reduced oxidative stress by increasing glutathione content. Treatment increased glutathione levels in the aqueous humour at timolol, bimatoprost and their fixed-dose combination, respectively ($p<0.05$) in Fig. 3. Furthermore, treatment with timolol, bimatoprost and their fixed-dose combination reduced glutamate levels in the vitreous humour ($p<0.05$) in Fig. 4.

Histological analysis of eyeballs: Histological analysis of some eyes (n = 5-6) was performed to describe the morphological alterations that occurred following cell injection. After injection, the size of the eyes began to increase four days later. In the eyes with raised IOP commencing 4 days following cell injection, the open-angle was blocked owing to iris adherence to the cornea. Figure 5a showed histological images of the control group displaying normal architecture of the anterior chamber and anterior synechiae with normal morphology of ciliary bodies showing no changes in IOP with a scale bar representing 5 mm. Figure 5b clearly defines the histological analysis of the cell-injected group demonstrating

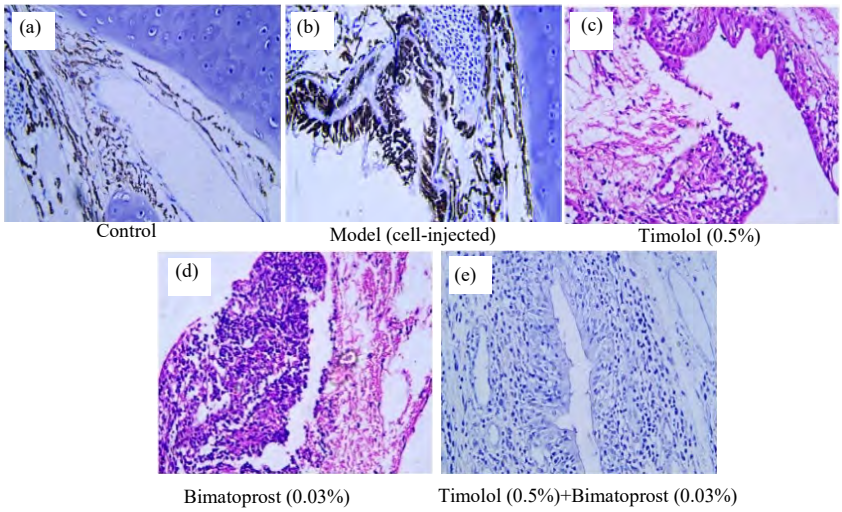

Fig. 5(a-e): Histological analysis of eyeballs (a) Histological analysis of the control, (b) Cell-injected, (c) Treatment group (0.5%, timolol, (d) Treatment groups (0.03% bimatoprost) and (e) Treatment groups of the combination (Bimatoprost 0.03%-timolol 0.5%), Representative images of the anterior chamber and anterior synechiae
Scale bars represent 5 mm

abnormal changes in the anterior chamber with inflamed ciliary bodies and angle-closure due to the adhesion of the iris to the cornea because of profound accumulation of conjunctival fibroblast injected intracamerally on the corneal endothelium and anterior synechiae with significant ($p<0.01$) IOP elevation with a scale bar representing 5 mm. Figure 5c shows histological analysis of treatment group with 0.5%, timolol with significant ($p<0.05$) reduction in intraocular pressure in the anterior chamber and reduced accumulation of conjunctival fibroblasts injected intracamerally on the corneal endothelium and anterior synechiae with a scale bar representing 5 mm. Figure 5d represents the histological analysis of the treatment group with 0.03% bimatoprost with significant ($p<0.05$) improvement in intraocular pressure with reduced inflammation of ciliary bodies in the anterior chamber and anterior synechiae induced by intracameral injection of conjunctival fibroblasts with a scale bar representing 5 mm. Figure 5e demonstrates the histological analysis of the combined treatment group of bimatoprost 0.03% and timolol 0.5% showing remarkable improvement ($p<0.01$) in intraocular pressure with reduced inflammation of ciliary bodies in the anterior chamber and anterior synechiae caused due to markedly decreased accumulation conjunctival fibroblast injected intracamerally. In Fig. 6a Histological micrographs showing normal architecture without any modifications in the Optic Nerve Head (ONH) cupping in the control group displaying no changes in IOP levels with a scale bar representing 200 µm. In Fig. 6b Histological micrographs showing the increase in the Optic Nerve Head (ONH) cupping observed after 16 days of cell injection intracamerally with elevated IOP with a scale bar representing 200 µm. In Fig. 6c, Histological micrographs showing significant ($p<0.05$) reduction in the Optic Nerve Head (ONH) cupping following treatment with 0.5%, timolol eye drops with decreased IOP with a scale bar representing 200 µm. Figure 6d Histological micrographs showing a significant decrease ($p<0.05$) in the Optic Nerve Head (ONH) cupping following treatment with 0.03% bimatoprost eye drops with decreased IOP with a scale bar representing 200 µm. In Fig. 6e, histological micrographs showing significant ($p<0.01$) reduction in the Optic Nerve Head (ONH) cupping following treatment with their fixed-dose combination of bimatoprost 0.03% and timolol 0.5% eye drop with a marked decrease in IOP with a scale bar representing 200 µm. Thus, therapy with hypotensive medications dramatically reduced morphological alterations such as lower IOP reduced iris-to-cornea adhesion and reduced ONH cupping. In Fig. 7a, histological micrographs of the control group showing the normal architecture of the retinal sections located 1 or 2 mm away from the optic nerve head with no changes in the retinal thickness and the Nerve Fiber Layers (NFL), Inner Plexiform Layers (IPL) and Inner and Outer Nuclear Layers (INL) with a scale bar representing 100 µm. In Fig. 7b, histological micrographs of retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was decreased after cell injection. It was also found that 8 days following cell injection, the Nerve Fiber Layers (NFL) and Inner Plexiform Layers (IPL) had started to shrink in thickness and that 16 days following cell injection, the retinae as well as its

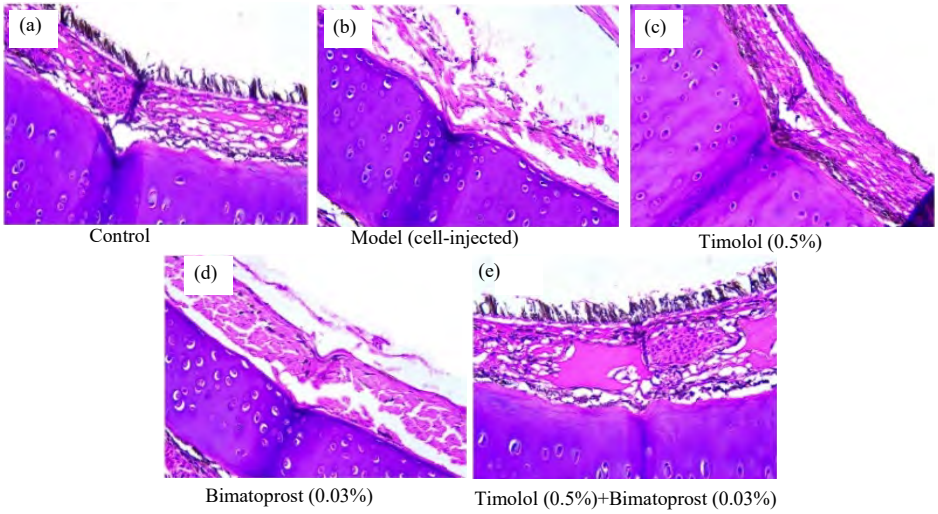

Fig. 6(a-e): Histological micrographs of optic nerve head (a) Histological micrographs of control showing Optic Nerve Head (ONH) cupping, (b) Histological micrographs of control showing Optic Nerve Head (ONH) cupping were observed 16 days after cell injection scale bars represent 200 μm, (c) Histological micrographs of control showing Optic Nerve Head (ONH) cupping following treatment with 0.5%, timolol, eye drops, (d) Histological micrographs showing reduced Optic Nerve Head (ONH) cupping following treatment with 0.03% bimatoprost eye drops and (e) Histological micrographs showing reduced Optic Nerve Head (ONH) cupping following treatment with 0.5%, timolol, eye drops
Scale bars represent 200 μm

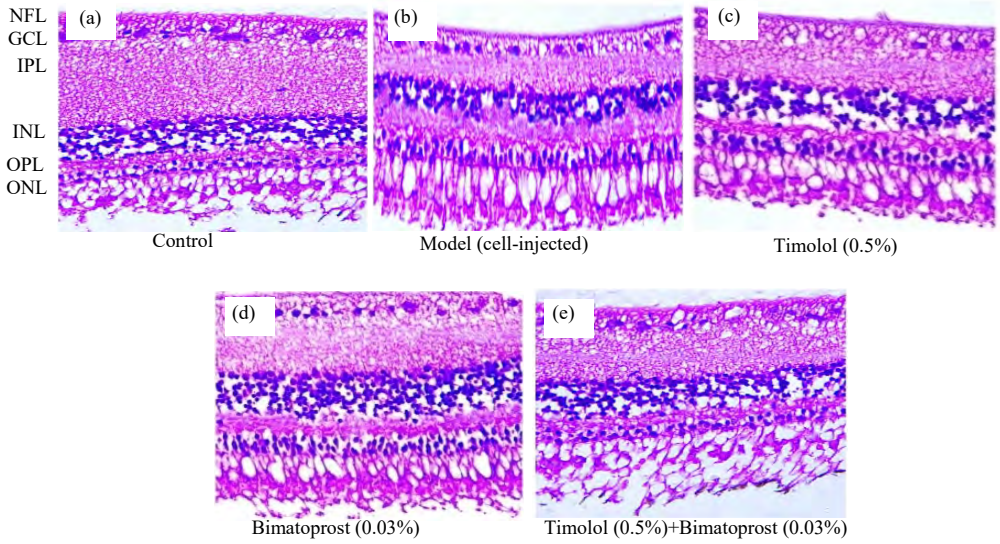

Fig. 7(a-e): Histological micrographs of retinal sections (a) Histological micrographs of the control groups showing retinal sections located 1 or 2 mm away from the optic nerve head, (b) Histological micrographs retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was decreased after cell injection, (c) Histological micrographs retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was improved remarkably after treatment with 0.5% timolol eyes drops, (d) Histological micrographs retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was improved remarkably after treatment with 0.03% bimatoprost eyes drops and (e) Histological micrographs retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was improved remarkably after treatment with (bimatoprost 0.03%-timolol 0.5%) eyes drops
Scale bars represent 100 μm. NFL: Nerve fiber layer, GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer

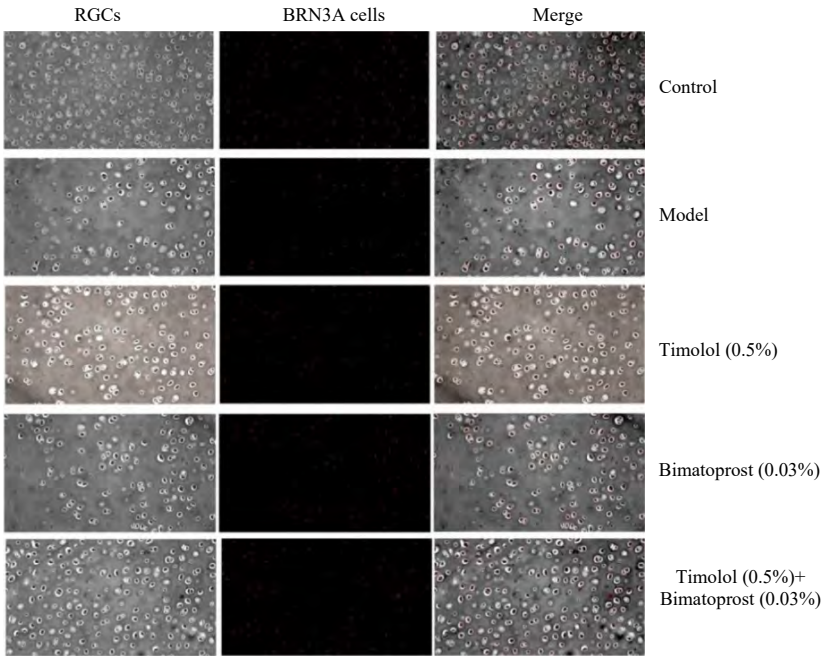

Fig. 8: Representation micrographs of RGCs in retinal field. Representation micrographs showing RGCs located 1 or 2 mm away from the optical nerve head. BRN3A-positive RGCs were identified on flat-mounted retinae. The number of RGCs decreased in the injected eyes and improved in treated groups (0.5%, timolol, 0.03%-timolol bimatoprost and their fixed-dose combination (bimatoprost 0.03%-timolol 0.5%) eyes drops

Scale bars represent 100 μm

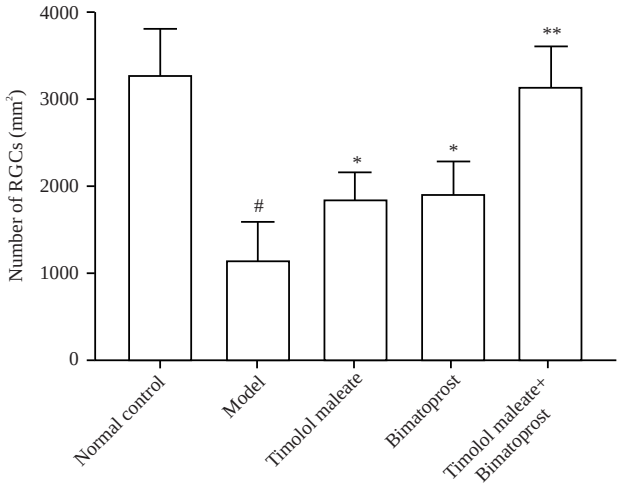

Fig. 9: Quantitative analysis of RGCs. The number of RGCs in the cell-injected eyes decreased compared to the control group on 16 days after cell injection. The number of RGCs was increased after treatment with 0.5%, timolol, 0.03% bimatoprost and their fixed dose combination (bimatoprost 0.03%-timolol 0.5%) eye drops

Data are presented as the Mean±SD. #p<0.01 compared control group, *p<0.05, **p<0.01 compared with the cell-injected group

Inner and Outer Nuclear Layers (INL) had shrunk in thickness with a scale bar representing 100 μm. In Fig. 7c, histological micrographs of retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was improved remarkably ($p<0.05$) after treatment with 0.5%, timolol eye drops. It was also found improvement in the thickness of the Nerve Fiber Layers (NFL), Inner Plexiform Layers (IPL) as well as its Inner and Outer Nuclear Layers (INL) following cell injection with a scale bar representing 100 μm. In Fig. 7d, histological micrographs of retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was improved remarkably ($p<0.05$) after treatment with 0.03% bimatoprost eye drops showing improvement in the thickness of the Nerve Fiber Layers (NFL), Inner Plexiform Layers (IPL) as well as its Inner and Outer Nuclear Layers (INL) following cell injection with a scale bar representing 100 μm. In Fig. 7e, histological micrographs of retinal sections located 1 or 2 mm away from the optic nerve head. The retinal thickness was improved remarkably ($p<0.01$) after treatment with the fixed-dose combination of bimatoprost 0.03% and timolol 0.5% eye drops showing improvement in restoring the thickness of the Nerve Fiber Layers (NFL), Inner Plexiform Layers (IPL) as well as its Inner and Outer Nuclear Layers (INL) to normal following cell injection after 4 weeks of repeated-dose administration with a scale bar representing 100 μm.

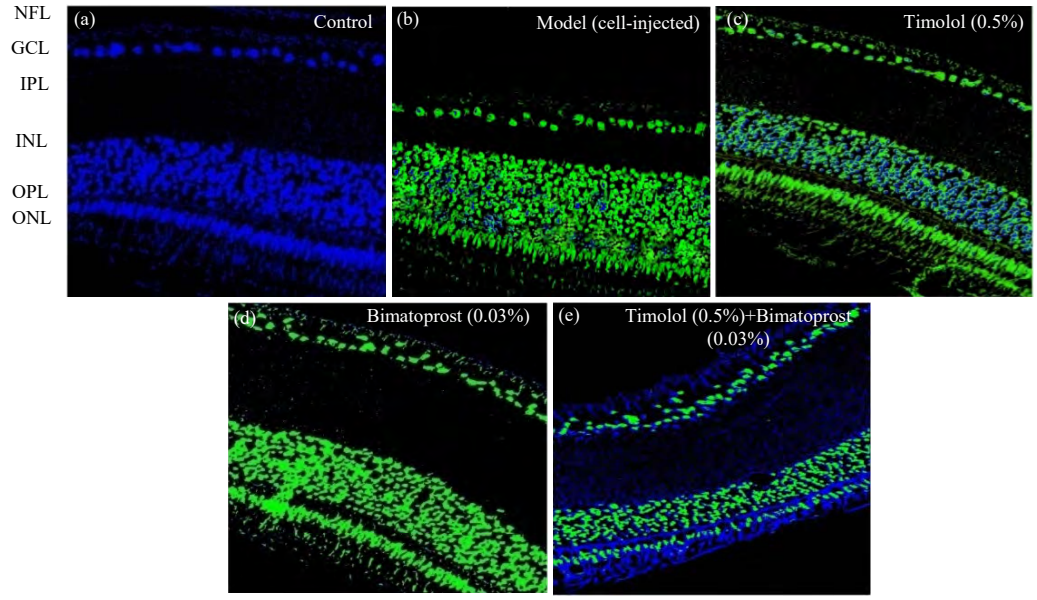

Fig. 10(a-e): TUNEL assay images of retinal ganglionic cells (RGCs) (a) TUNEL assay for retinal cells of control group. Representative images of TUNEL-stained retinal sections 2 mm away from the optic nerve head. Nuclei of cells were stained with DAPI (blue), (b) TUNEL assay for retinal cells. Representative images of TUNEL-stained retinal sections 2 mm away from the optic nerve head. TUNEL positivity was observed in the GCL, INL and ONL of retinae in the injected eyes 16 days after cell injection. Positive controls were treated with DNase I to induce DNA fragmentation. Nuclei of cells were stained with DAPI (blue), (c) TUNEL assay for retinal cells. Representative images of TUNEL-stained retinal sections 2 mm away from the optic nerve head. TUNEL positivity was observed in the GCL, INL and ONL of retinae in the injected eyes 16 days after cell injection but restored to normal following treatment with 0.03% bimatoprost eye drops. Positive controls were treated with DNase I to induce DNA fragmentation. Nuclei of cells were stained with DAPI (blue), (d) TUNEL assay for retinal cells. Representative images of TUNEL-stained retinal sections 2 mm away from the optic nerve head. TUNEL positivity was observed in the GCL, INL and ONL of retinae in the injected eyes 16 days after cell injection but restored to normal following treatment with their fixed dose combination (bimatoprost 0.03%-timolol 0.5%) eye drops. Positive controls were treated with DNase I to induce DNA fragmentation. Nuclei of cells were stained with DAPI (blue) and (e) TUNEL assay for retinal cells. Representative images of TUNEL-stained retinal sections 2 mm away from the optic nerve head. TUNEL positivity was observed in the GCL, INL and ONL of retinae in the injected eyes 16 days after cell injection but restored to normal following treatment with their fixed dose combination (bimatoprost 0.03%-timolol 0.5%) eye drops. Positive controls were treated with DNase I to induce DNA fragmentation. Nuclei of cells were stained with DAPI (blue)

Scale bars represent 100 μm. GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer

Quantitative analysis of RGCs: The ganglion cell nuclear protein expression (BRN3A) is explicitly in the ganglion cell nuclei, therefore, BRN3A-positive cells were regarded as retinal ganglionic cells in Fig. 8 and 9. Treatment with hypotensive drugs, on the other hand, significantly ($p<0.01$) increased the BRN3A-positive cells after a repeated dose of drug administration for 4 weeks.

The average number of RGCs on the 4th, 8th and 16th day after IOP elevation was 3456±256, 1857±451 and 921±216 cells mm^{-2} (n = 5-9), in model group (cell-injected eyes) with raised IOP and 3045±214, 2529±234 and 2212±130 cells mm^{-2} (n = 5-9), respectively in the control group in Fig. 9. The count of RGCs was considerably lesser in the model group at 16 days following cell injection relative to the control group ($p<0.01$). Nevertheless, the treatment with hypotensive drugs significantly ($p<0.01$) increased the mean number of RGCs after repeated-dose administration.

TUNEL assay of Retinal Ganglionic Cells (RGCs): The assay was used to evaluate the loss of retinal ganglionic cells following injection. The model group in Fig. 10(a-e) had more

TUNEL positive cells in the ONL, INL and GCL than the control group (16 days after cell injection). However, timolol, bimatoprost and their fixed-dose combination reduced the number of TUNEL-positive cells.

DISCUSSION

Glaucoma is an eye disease that damages the optic nerves, exacerbating irregular eye pressure[31]. It is a leading cause of visual impairment in people over 60 worldwide[32]. Glaucoma is caused by vascular dysregulation and increased intraocular pressure[33] and that this damage in the retinal ganglion cells is the primary step in the disease's progression[34]. Apoptosis and neuronal damage in the Retinal Ganglion Cell (RGC) appear to be caused by a variety of factors, including a lack of neurotrophic supply, the generation of neuronal NO (nitric oxide) synthesis, a decreased blood flow to the ONH, glutamate-induced excitotoxicity and generation of extracellular ions and neurotoxic reactive oxygen substances[35-37]. Neuroprotective effects of glaucoma drugs namely, bimatoprost and adrenergic antagonists were studied. A significant *in vitro* neuroprotective effect of bimatoprost was demonstrated in cellular models of neuronal degeneration[38]. In addition, it was shown to have a neuroprotective effect in *in vivo* optic nerve degeneration models[39], endothelin-1-induced optic nerve ischemia[40], chronic ocular hypertension, transient ischemia as well as photoreceptor degeneration[41]. Adrenergic antagonists, namely nipradilol, timolol, betaxolol, carteolol and levobetaxolol were found to be efficacious offering neuroprotection by inhibiting increase uptake of intracellular calcium ions,[42] blockages of sodium channels,[43] promoting the release of brain-derived neurotrophic factors[44] and having the potential to exert action as free-radical scavengers in animal studies.

It is a combination of the drugs, bimatoprost 0.03% timolol 0.5%, which has an additive effect on reducing intraocular pressure (IOP). Both drugs reduce intraocular pressure (IOP) by preventing aqueous humour formation, accomplished through a diverse molecular level mechanism of action. However, when administered together, they show an additive effect[24,25].

A combined study of 3-month involving 1000 patients with ocular hypertension or glaucoma showed the prevalence of adverse events related to the treatment with bimatoprost and timolol, which was substantially lower when compared to bimatoprost alone (40.1 vs 51.2%, $p<0.001$)[27].

Bimatoprost/timolol was considered to be substantially more efficacious than latanoprost and timolol combination in lowering average diurnal IOP and IOP at each time-point determined in patients of four weeks study with higher IOP who were not adequately controlled with prostamides/prostaglandin analogs monotherapy ($p<0.001$). Bimatoprost/timolol significantly reduced IOP at 09: 00 hours more than latanoprost/timolol (2.9 vs 1.9 mmHg, $p = 0.002$)[45].

A study conducted on patients with open-angle glaucoma found that topical bimatoprost/timolol improved several parameters of ocular blood flow[46-49]. It appears that the increased activity of both components is effective in ocular hemodynamic variables observed with bimatoprost and timolol. It was shown that bimatoprost reduces the level of arteriovenous outflow and improves both intraocular and retrobulbar hemodynamic variables in glaucoma.

Numerous studies have found that timolol possesses both, IOP lowering and neuroprotective effects[50, 51]. These findings suggest the evidence of bimatoprost/timolol, a combination of both components, having a neuroprotective effect. However, no reports of such fixed-dose combination having a neuroprotective effect were studied so far, on the conjunctival fibroblasts induced chronic glaucoma rat model. Thus, assessing the neuroprotective effects of a marketed available combination drug for the therapy of glaucoma may be beneficial[52].

A rat chronic hypertensive model was developed in this study using a method that we had previously developed and validated in other studies[53]. When the cells were injected intracamerally into the anterior chamber of rats 4 days following cell injection, the intraocular pressure was increased significantly compared to that of control eyes. The highest IOP was noticed on the seventh day. The levels of the IOP profile persisted above normal for the next 16 days following cell injection. A high IOP was achieved in 76% of the cell-injected eyes in our model. This finding demonstrates that in the current rat model, a sustained increase in intraocular pressure (IOP) could be induced. Slit-lamp examination demonstrated cell aggregation onto the endothelium of the cornea and anterior synechiae beginning 4 days following cell injection. These modifications eventually resulted in the blockage of the aqueous outflow, with an increase in IOP. The combination of bimatoprost (2%) and timolol (0.5%) had a significant ($p<0.05$) IOP-lowering effect in the chronic glaucoma rat model, with the marked restoration of aqueous outflow and reduction of cell aggregation on the corneal endothelium and anterior synechiae after repeated-dose administration.

However, RGC loss was observed in the model group 16 days after cell injection. This finding suggests that chronically high IOP caused IOP-dependent cell death, emulating the clinical pathogenesis of glaucoma. RGC staining

was considerably improved in the rat retina after Bimatoprost 2 percent/timolol 0.5% treatment, demonstrating a protective effect in chronic ocular hypertensive eyes. Therefore, by reducing the RGC loss, treatment with timolol, bimatoprost and their combination may offer neuroprotective drug treatments for glaucoma.

The model group demonstrated glaucoma-specific features such as retinal thinning and augmented cupping of ONH. Mechanical contraction caused by the elevation of IOP distorts the glial lamina in rats or lamina cribrosa in humans, resulting in RGC dysfunction in glaucoma[54,55]. The current model also showed histological and morphological alterations that resulted in retinal thinning and excessive ONH cupping. These findings corroborate the hypothesis that optic neuropathy is triggered by the compression of ONH as a result of IOP rise in a rat model, which is analogous to changes in glaucoma. As a result, the eyeballs' size with raised IOP, leading to the prediction that the whole posterior retina would be exposed to a risk of high pressure. Furthermore, treatment with timolol, bimatoprost and their combination restored the histological and morphological changes dramatically, improving the retinal thickness with deceased ONH cupping, implying that hypotensive drugs have a neuroprotective effect.

TUNEL-positive cells were seen 7 days following cell injection in the GCL, INL and ONL. Current findings are in agreement with previous clinical observations showing that the retinae of glaucoma patients are loaded with TUNEL-positive cells. Hypotensive drugs reduced the number of TUNEL-positive cells after a repeated drug administration. Current study findings showed the potential of investigating on conjunctival fibroblasts induced chronic glaucoma rat model for further research into the pathogenic basis of glaucoma and thus paving new avenues for developing IOP lowering and neuroprotective drugs.

CONCLUSION

Current findings demonstrated a substantial reduction in the loss of RGC following the administration of (0.03%) bimatoprost and (0.5%) timolol in a chronic glaucoma rat model induced by intracamerally injected conjunctival fibroblasts. The combination of bimatoprost (0.03%) and timolol (0.5%) showed a protective effect against retinal injury in the TUNEL assay. As a result, it was concluded that the combination of bimatoprost (0.03%) and timolol (0.5%) percent offers neuroprotective prospects for the treatment of glaucoma. The study findings suggest that the reduction of IOP offered by the bimatoprost (0.03%) and timolol (0.5%) may be implicated in the decrease of RGC loss in the management and treatment of glaucoma.

SIGNIFICANCE STATEMENT

This study discovered the effects of bimatoprost (0.03%) and timolol (0.5%) that can be beneficial for the treatment of glaucoma. This study will help the researchers to uncover the critical areas of treatment strategies for a chronic model of glaucoma in rats induced by intracameral injection of conjunctival fibroblasts that many researchers were not able to explore. Thus a new theory on glaucoma treatment may be arrived at to understanding the management and treatment strategies in a novel chronic model of glaucoma in rats.

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