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Research Article

Protective Effect of Fangchinoline Against Glaucoma and Neuroinflammation in Unilateral Ocular Hypertension in Mice

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Abstract

Background and Objective: Glaucoma is an optic nerve injury caused by a rise in intraocular pressure. Conventional drugs are available for the management of the disorder but do not prevent it, so there is a need to find novel therapies for it. The current study assesses the efficacy of fangchinoline in treating glaucoma and speculates on the proposed mode of action. **Materials and Methods:** Unilateral ocular hypertension was induced by exposing the mice to laser and administering fangchinoline 10 and 30 mg kg⁻¹ i.p., for 1 week. Intraocular pressure was measured after the 1, 2, 3, 5 and 7 days of laser exposure. The level of oxidative stress markers, inflammatory cytokines and glutamate were estimated in glaucoma animals. Microglia and RGCs count were done in the retinal tissue of glaucoma mice. **Results:** The fangchinoline-administered group expressed a substantial reduction in IOP when compared to an untreated control group of animals. The animals administered fangchinoline had lower levels of inflammation-related cytokines and oxidative stress in their aqueous humour than the animals in the negative control group. The glutamate level, RGC and microglia count were all higher in the group of animals given fangchinoline compared to an untreated control group. **Conclusion:** According to the findings in the investigation, fangchinoline therapy prevents glaucoma in mice with unilateral ocular hypertension by lowering neuroinflammation and oxidative stress.

Key words: Fangchinoline, ocular hypertension, neuroinflammation, glaucoma, oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Glaucoma is a clinical disease characterised by optic nerve injury caused by a rise in intraocular pressure and it is a significant source of permanent blindness. Literature reveals that in 2040 approximately 100 million patients suffer from glaucoma between the age of 40-80 years¹. Glaucoma is exacerbated by intraocular pressure, which causes retinal ganglion cell and optic nerve injury, leading to a progressive decrease in the vision field². In glaucoma, there are multiple pathogenic causes, including excitotoxicity-induced neuronal destruction and oxidative injury to the optic nerve.

Currently, available medicine majorly targets IOP for the management of glaucoma, which does not prevent neurodegeneration. However, neurodegeneration associated with glaucoma is irreversible. Literature reveals that neurodegeneration associated with glaucoma occurs due to the activated immune cells as activation of microglia is responsible for the neuroinflammation of CNS³. In the experimental model (unilateral and laser-induced ocular hypertension in the animal model), human loss of RGC leads to the activation of microglia cells⁴. Management of glaucoma needs to target neurodegeneration and thus the development of neuroprotective therapy for the effective treatment of glaucoma.

In the recent era, phytochemicals have gained attention for the discovery of alternate medications for the treatment of chronic diseases, such as glaucoma. Fangchinoline is a phytochemical characterised as an alkaloid derived from *Stephania tetrandra*⁵. Several potential medicinal properties such as cytotoxic, anti-inflammatory and antioxidant are reported for fangchinoline⁶⁻⁸. Moreover, fangchinoline was studied for its antirheumatic, anti-osteoporotic, anti-cancer and cardioprotective activity^{6,9-11}. It was also suggested for neuroprotective activity by reducing inflammatory mediators and oxidative stress¹² and also reported for the treatment of retinopathy¹³. Therefore, the current study demonstrated the effects of fangchinoline on glaucoma-protective function.

MATERIALS AND METHODS

Study area: The study was carried in the First People's Hospital of Zigong, China during the period of June to September, 2021.

Animals: In this study, 24 Swiss albino mice (sex: male, weight: 40-50 g, age: 12-14 weeks) were housed under a controlled condition (humidity: 60±5%, temperature: 25±0.5°C, 12 hrs light/dark cycle) as per the reported

guidelines. The study methodology was authorised by the institutional ethical council (Reg. No. IACUC/FPHZ/2020/05).

Experimental: A unilateral ocular hypertension-induced glaucoma model was developed by exposing one eye of mice with a single session of the laser as studies by Fu and Sretavan¹⁴. All mice were anaesthetized and directly exposed to the episcleral and limbal veins with laser without using any lens. Laser light of 0.3 W output and 50-100 µm size was used for the period of 0.5 sec. Approximately 55-76 burns were received by each eye of the mice. Further tonometer was used to assess the IOP in both lasers treated and the collateral eye of mice. All the animals were split into 4 groups (n = 6): Control, negative control and fangchinoline 10 and 30 mg kg⁻¹ administered (i.p.). The IOP was determined after the 1, 2, 3, 5 and 7 days of laser exposure.

Glutamate estimation: Glutamate level in the vitreous humour was estimated using a commercially available kit. Each mouse's vitreous humour was removed and sonicated with 0.2 M perchloric acid. The mixture was centrifuged at 15000 g for 5 min. The glutamate concentration was determined using the supernatant. Collected sample was then treated with kit chemicals as per the instruction and absorbance were determined at 405 nm using UV spectroscopy.

Determination of oxidative stress parameters: Aqueous humour was collected from the eyes of each mouse by puncturing the anterior chamber with a needle 30 gauge. Later aqueous humour was deproteinized by treating with metaphosphoric acid and triethanolamine and then collected aqueous humour was used for the determination of oxidative stress parameters such as SOD and MDA as per the instruction of the manufacturer of their kits.

Assessment of inflammatory cytokines: Inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the aqueous humour of unilateral ocular hypertension induced glaucoma mice model as per the direction of manufacturer of the kit using ELISA.

Assessment of RGCs count: All the animals were euthanized and eyeballs were isolated after the cardiac perfusion, later fix the eyeball tissue with 4% paraformaldehyde solution at 4°C for 1 hr. Anterior segment of the eye was isolated and fix it for a further 7 hrs, retinal tissue was radially cut into four pieces which were removed from the microscopy. Coverslip was applied on retinal tissue and kept the same at room

temperature overnight under 10 g weight pressure. Coverslip was removed and tissue was dropped with 1% cresyl violet solution (100 μ L), tissue was immersed in PBS for 10 min each time 3 times after keeping it at 37°C for 2 hrs. Alcohol was applied to dehydrate the tissue and transparency by xylene and the tissue was observed under a light microscope.

Nissl staining was applied and the RGC layer was observed for characteristics of cell morphology and RGC characteristics were determined as follows such as abundant cytoplasm, clear nucleolus and nucleus, round and large cell body. The cells with the aforementioned characteristics would be considered RGCs.

Estimation of microglia count: The retina was isolated from each animal and retinal tissue was sectioned radically into four and three-centric regions. The retina of each eye was mounted and the microglia were counted in the superficial layer of the retina.

mRNA expression of NF- κ B and TNF- α : The levels of NF- κ B and TNF- α in the mRNA were determined using qRT-PCR. Total mRNA was removed from retinal cells by utilizing the TRIzol reagent (Thermo Fisher Scientific). The oligo (dT) 15 primer was utilised to generate cDNA from RNA by using M-MLV reverse transcriptase. The mixed solution was kept at 42°C for 15 min before being disabled by warming it for 5 sec at 85°C and eliminating the gDNA. The qPCR system had a total volume of 20 μ L, which included forward (0.4 μ L) and reverse primers (10 μ mol L⁻¹), 2 TransStart® Tip Green qPCR Supermix (10 μ L), cDNA template (1 μ L) and enough H₂O.

In PCR, the denaturation process was carried out for 30 sec at 94°C, 5 sec at 94°C, 15 sec at 60°C and 10 sec at 72°C for 45 cycles. The specimen's CT levels were calculated and expression levels were expressed by 2^{- $\Delta\Delta$ CT}.

Statistical analysis: The results were expressed as a mean SEM (n = 10). Statistically, differences between animal cohorts were assessed using SPSS software (ver. 13.0, USA) and a one-way analysis of variance with Tukey's *post hoc* analysis was used. A significant threshold of p<0.05 was maintained.

RESULTS

Assessment of fangchinoline on intraocular pressure: In unilateral ocular hypertension-induced glaucoma mice, IOP was determined for a period of 7 hrs using a tonometer as expressed in Fig. 1. The negative control group had a considerably (p<0.01) higher IOP than the control group. Furthermore, IOP was considerably (p<0.01) lower in the fangchinoline-administered group compared to the negative control group of animals, according to the dose-dependent approach.

Analysis of fangchinoline on the level of glutamate: The level of glutamate was assessed in the vitreous humour of animals with unilateral ocular hypertension-induced glaucoma induced by fangchinoline (Fig. 2). The glutamate level in the negative control group was lower than in the control group of animals. Furthermore, administration with fangchinoline improved the vitreous humour of animals with unilateral ocular hypertension-induced glaucoma.

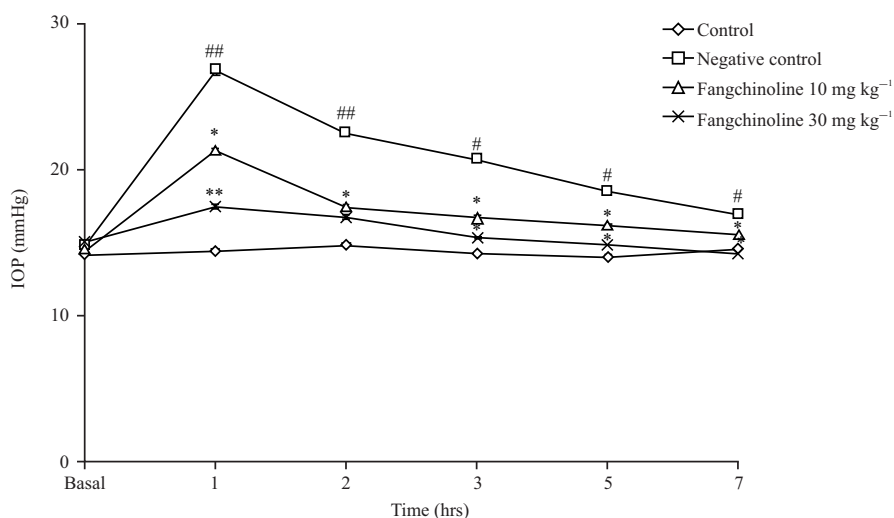


Fig. 1: Effect of fangchinoline on intraocular pressure (IOP) of unilateral ocular hypertension in mice
Mean $\pm$ SEM (n = 10), #p<0.05 and ##p<0.01 compared to control group, *p<0.05 and **p<0.01 compared to the negative control group

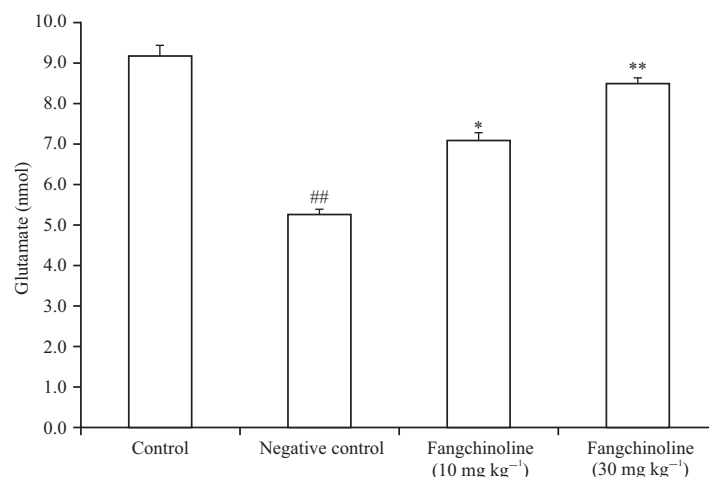


Fig. 2: Effect of fangchinoline on the level of glutamate in the vitreous humour of unilateral ocular hypertension induced glaucoma in mice

Mean $\pm$ SEM (n=10), ##p<0.01 compared to control group, *p<0.05 and **p<0.01 compared to the negative control group

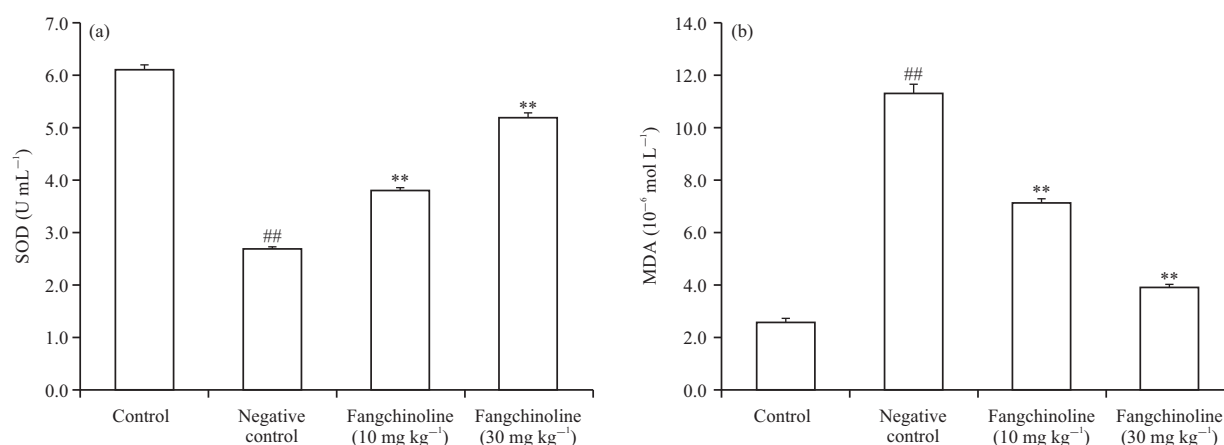


Fig.3(a-b): Effect of fangchinoline on the oxidative stress in aqueous humour of unilateral ocular hypertension induced glaucoma in mice, (a) Level of SOD and (b) Level of MDA in aqueous humour of unilateral ocular hypertension induced glaucoma in mice

Mean $\pm$ SEM (n = 10), ##p<0.01 compared to control group and **p<0.01 compared to the negative control group

Analysis of fangchinoline on the markers of oxidative stress:

Oxidative stress markers such as SOD and MDA were determined in the aqueous humour of fangchinoline-administered unilateral ocular hypertension-induced glaucoma. The SOD levels were expressed in Fig. 3a and the MDA levels were shown in Fig. 3b. The SOD and MDA levels were considerably lower and greater in the aqueous humour of the negative control group than in the animal control group, respectively (p<0.01). Fangchinoline attenuated the altered levels of SOD and MDA in the aqueous humour of animals with unilateral ocular hypertension-induced glaucoma.

Analysis of fangchinoline on inflammatory cytokines:

In the retinal tissue homogenate of animals with unilateral ocular hypertension-induced glaucoma, the effect of fangchinoline on inflammatory cytokines such as IL-1, IL-6 and TNF- α was studied. The levels of IL-1, IL-6 and TNF- α in the retinal homogenate of the negative control group were significantly higher than in the control group of animals. In the retinal tissue of the animals administered fangchinoline, there were considerably fewer inflammatory cytokines than in the animals in the negative control group (Fig. 4).

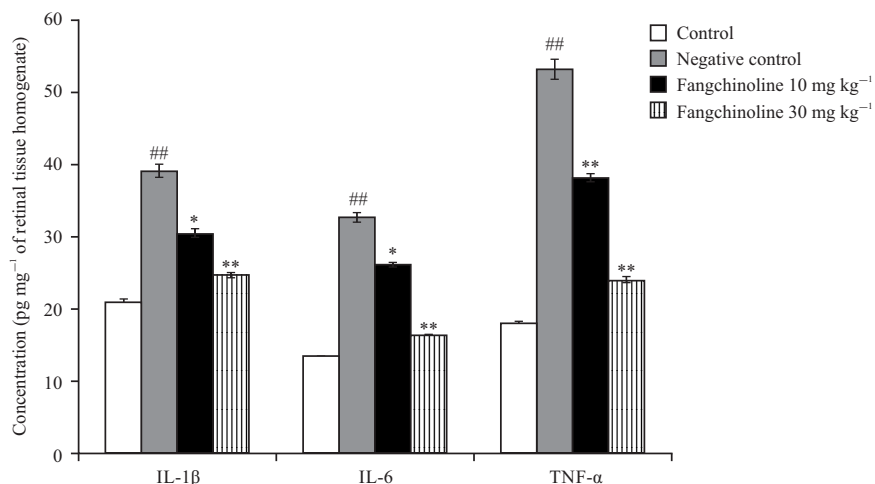


Fig. 4: Effect of fangchinoline on inflammatory cytokines such as IL-1β, IL-6 and TNF-α in retinal tissue homogenate of unilateral ocular hypertension induced glaucoma in mice

Mean ± SEM (n = 10), ##p < 0.01 compared to control group, *p < 0.05 and **p < 0.01 compared to the negative control group

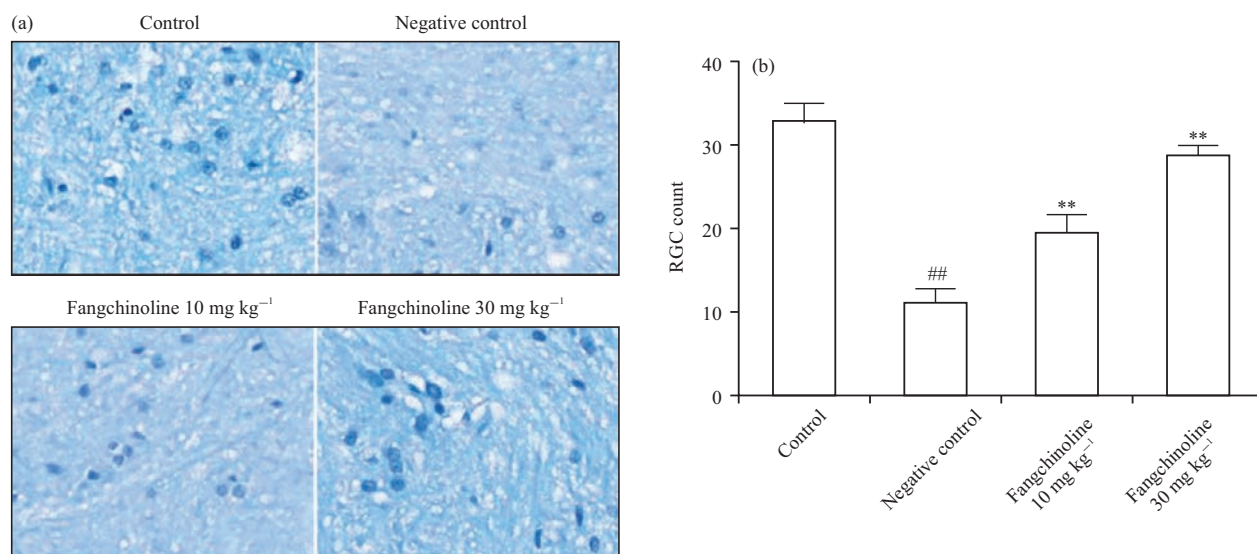


Fig. 5(a-b): Effect of fangchinoline on RGC count in retinal tissue of unilateral ocular hypertension induced glaucoma in mice, (a) Micrograph of the retinal tissue shows control, negative control, fangchinoline (10 and 30 mg kg⁻¹) administered retinal tissue and (b) RGC count in retinal tissue of unilateral ocular hypertension-induced glaucoma in mice

Mean ± SEM (n = 10), ##p < 0.01 compared to control group and **p < 0.01 compared to the negative control group

Analysis of fangchinoline on RGCs count: The RGC count was observed in fangchinoline-administered retinal tissue homogenate of unilateral ocular hypertension-induced glaucoma in animals (Fig. 5). Micrographs of control, negative control and fangchinoline (10 and 30 mg kg⁻¹) administered retinal tissue were shown in Fig. 5a. The negative control group of animals demonstrated a significant drop in RGC count in the retinal tissues as compared to the control group. The RGC count in the retinal tissue of the fangchinoline-

administered group increased more than that of the negative control group as per the dose-dependent manner (Fig. 5b).

Analysis of fangchinoline on relative mRNA expression of NF-κB and TNF-α: Relative mRNA expression of NF-κB and TNF-α in retinal tissue of fangchinoline-treated unilateral ocular hypertension-induced glaucoma in animals (Fig. 6). The relative mRNA expression of NF-κB and TNF-α in the negative control group's retinal tissue was substantially greater

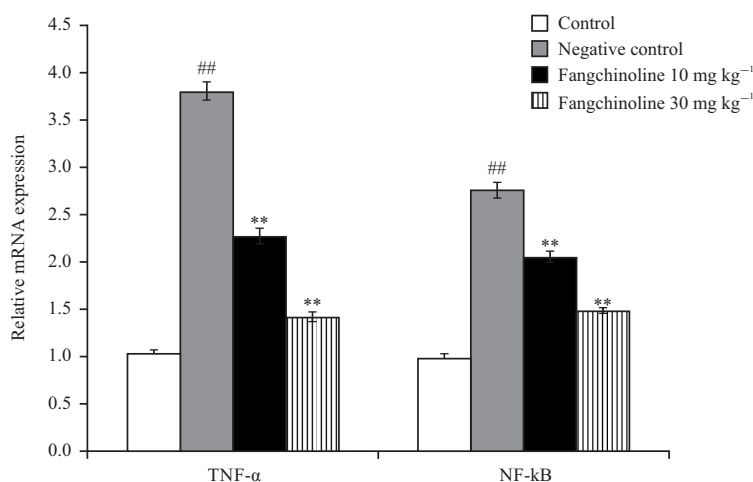


Fig. 6: Effect of fangchinoline on relative mRNA expression of NF-κB and TNF-α in retinal tissue of unilateral ocular hypertension induced glaucoma in mice

Mean ± SEM (n = 10), ^{##}p<0.01 compared to control group and ^{**}p<0.01 compared to the negative control group

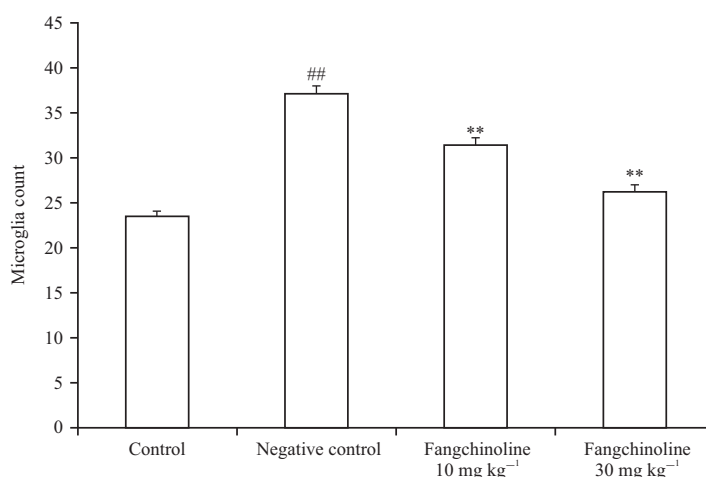


Fig. 7: Effect of fangchinoline on microglia count in retinal tissue of unilateral ocular hypertension induced glaucoma in mice

Mean ± SEM (n = 10), ^{##}p<0.01 compared to control group and ^{**}p<0.01 compared to the negative control group

(p<0.01) than in the control group of animals. This study proved that administration of fangchinoline can alter the mRNA expression of NF-κB and TNF-α in the retinal tissue of animals with unilateral ocular hypertension-induced glaucoma.

Analysis of fangchinoline on microglia count: The impact of fangchinoline was observed on the microglia count in the retinal tissue of mice with unilateral ocular hypertension-induced glaucoma (Fig. 7). The number of microglia was considerably greater (p<0.01) in the negative control group's retinal tissue than in the control group's animals. When compared to a negative control group of animals, the

fangchinoline-administered group had a substantial drop in microglia count in the retinal region.

DISCUSSION

Glaucoma is a chronic ophthalmic disorder in which an increase in intraocular pressure damages the optic nerve. Clinically, glaucoma is a significant source of blindness in those over the age of 60¹⁵. The IOP increases in glaucoma, which promotes injury to retinal cells. Literature suggests that excitotoxicity, neuroinflammation and oxidative stress are the possible molecular pathogenesis involved in the development of glaucoma¹⁶. Effective management of glaucoma is achieved

by targeting these molecular approaches to glaucoma treatment. As a result, the current study investigates the impact of fangchinoline on glaucoma in a unilateral ocular hypertension animal model.

Glaucoma is clinically diagnosed by an increase in IOP and the drug used to reduce it is used for the effective management of it¹⁷. According to the results of this research, IOP was lowered more in the fangchinoline-administered group than in the negative control group. Oxidative stress contributes to the development of neuroinflammation and excitotoxicity, which damage the optic nerve in glaucoma¹⁸. Unilateral ocular hypertension enhances oxidative stress as it induces hydroxyl radicals and superoxide anions in the retinal tissues of glaucoma mice¹⁹. The present study found that oxidative stress reduces glutamate levels in the vitreous humour, which contributes to optic nerve damage²⁰. Treatment with fangchinoline ameliorates the markers of oxidative stress and glutamate in unilateral ocular hypertension-induced glaucoma in mice. These changes lead to improved inflammatory cytokines, which contribute to neuronal inflammation. According to the current study, the negative control group had greater levels of cytokines than the control group of animals and fangchinoline therapy restored them. Microglia in the CNS have been known to provide immunoprotection and an increase in their count activates the immune reaction that causes neuroinflammation²¹. Literature suggested that microglia count is responsible for neuroinflammation in glaucoma patients, which leads to optic nerve damage too²². In comparison to a negative control group of animals, the number of microglia in the retinal tissue of the fangchinoline-treated group was significantly reduced.

Literature revealed that in glaucoma due to an increase in IOP and neuroinflammation loss of RGCs count occurs²³. Retinal ganglionic cells are responsible for normal vision and loss of RGCs count contributes to loss of vision²⁴, data from the study also supported it. When compared to a negative control group of animals, the fangchinoline-treated group shows a drastically decreased number of microglia in the retinal tissue.

CONCLUSION

Fangchinoline administration prevents glaucoma in unilateral ocular hypertension animals by reducing neuroinflammation and oxidative stress. The study's findings implied that fangchinoline might be utilized therapeutically to treat glaucoma.

SIGNIFICANCE STATEMENT

Glaucoma is a clinical disease that causes optic injury due to an increase in intraocular pressure and leads to lifelong blindness. This disease primarily affects the elderly. Therefore, there is a need to find novel therapies for the treatment of retinopathy. The current investigation assesses the efficacy of fangchinoline in treating glaucoma and speculates on its mechanism of action. The outcomes of this study revealed that fangchinoline administration prevents glaucoma in unilateral ocular hypertension animals by reducing neuroinflammation and oxidative stress. The study's findings imply that fangchinoline might be used therapeutically to treat glaucoma.

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