

INTRAOCULAR PRESURE LOWERING EFFECTS OF 0.75% SILIBININ DIHEMISUCCINATE EYE DROPS IN RABBITS MODEL OF α -CHYMOTRYPSIN-INDUCED GLAUCOMA

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ABSTRACT

Although glaucoma is no longer defined as elevated intraocular pressure (IOP) but rather a condition comprises characteristic optic nerve head and visual field abnormalities, lowering IOP is still the major strategy in slowing down glaucomatous damage to the inner structures of the eye and visual field. The present study was designed to evaluate the IOP lowering effect of a novel eye drops formula of 0.75% silibinin dihemisuccinate (SDH) in α -chymotrypsin-induced glaucoma in rabbits. Sixty New Zealand white rabbits were used in this study and allocated into different groups; the effects of single dose and long term use of SDH drops on IOP in rabbits with α -chymotrypsin-induced glaucoma were evaluated using two types of tonometry. The results showed that SDH drops significantly lowered IOP when topically instilled both as short or long-term treatment in this animal model compared to the already utilized drugs used in this respect like Betaxolol or Pilocarpine. In conclusion, the novel formula of SDH eye drops was effective in management of glaucoma by decreasing IOP, probably through interfering with both inflow and outflow of aqueous humor dynamics, and it may be a new drug candidate for the reduction of elevated IOP.

Keywords: Silibinin, glaucoma, eye drops, IOP.

INTRODUCTION

Although glaucoma is no longer defined as elevated intraocular pressure (IOP) but rather a condition comprises characteristic optic nerve head and visual field abnormalities¹, lowering IOP is still the major strategy in slowing down glaucomatous damage to the inner structures of the eye and visual field.² All current treatment strategies are designed to reduce IOP by reducing the rate of aqueous humor (AH) formation and/or enhance its drainage out of the eye.³ The ciliary epithelium has α 2- and β 2-adrenergic receptors. Stimulation of α -receptors or inhibition of β 2-receptors was thought to reduce AH formation.⁴ Topical Epinephrine administration decreases the rate of AH formation, an effect thought to be mediated by β -receptor induced increase in cAMP in the ciliary epithelium.⁵ The participation of cAMP in this effect has been supported by finding that activators of adenylcyclase (cholera toxin and Forskolin) decrease AH formation and hence IOP in experimental animals and human.⁶ Targeting of this Cl⁻ transport system is thought to be the newer proposed mechanism for the lowering of IOP by the oldest antiglaucomatous drug, Timolol.⁷ These findings support the major involvement of increased rather than decreased cAMP as a second messenger mechanism in the control of AH formation in normal physiology, as well as in

pathological conditions. Interestingly, the action of β -blockers in the reduction of AH formation is now suggested to involve cAMP-independent mechanism.⁷ Furthermore, Timolol was shown to reduce Epinephrine-induced increase in uveoscleral outflow when the two drugs applied concurrently.⁸ Inhibition of PDE by flavonoids has been previously described^{9,10}; Silibinin, a powerful antioxidant flavonoids¹¹, has been shown to reduce IOP in normotensive rabbits when used alone as a solution in arachis oil in different concentrations, with greater effect achieved with 0.75% dose¹². Recently, we have reported a decrease in IOP of normotensive rabbits after ocular instillation of oily Silibinin Dihemisuccinate (SDH) solution.¹³ The site of action did not exactly pointed but the drug shown to delay IOP recovery rate after i.v. infusion of 20% NaCl solution. This largely suggests an interference with AH inflow mechanism. Interestingly, SDH was shown to inhibit cAMP-phosphodiesterase enzyme more potent than Theophylline or Papaverine.¹⁴ The present study was designed to evaluate the IOP lowering effect of a new eye drops formula of 0.75% SDH in α -chymotrypsin-induced glaucoma in rabbits, and compare the effect with the currently used drugs for management of elevated IOP in glaucoma.

MATERIALS AND METHODS

Chemicals

Silibinin Dihemisuccinate in pure form was purchased from Tolbiac S.R.L. (Argentina) and all other chemicals were purchased from the specialized supplying companies

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(as mentioned in methodology) and stored in the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad. Silibinin Dihemisuccinate was specially formulated as an eye drops suspension form (0.75%). Betaxolol 0.5% (Alcon, Cham, Switzerland) and Pilocarpine 2% (EPICO, Cairo, Egypt) were used as commercial eye drops formulas.

Animals

Sixty New Zealand white rabbits weighing 2.5-3.5 kg were used in this study, and treated according to the ethics of animal experiments approved by the University of Baghdad. Animals were kept in the animal house of the College of Pharmacy, University of Baghdad, under standardized conditions (12 hrs light-dark cycles at room temperature) and were fed standard diet and given water *ad libitum*.

Alpha-Chymotrypsin Glaucoma Model

Ocular hypertension was induced in the right eye as previously described.¹⁶ Briefly, New-Zealand albino rabbits of both sexes (Purchased from the local breeding marker according to the specifications of the National Center for Drug Research and Quality Control) weighing 2.5-3.5 kg were housed under controlled and standardized conditions of temperature, humidity and 12:12 hr light/dark cycle. Animals were identified with a tattoo in the ear and maintained on a 12 hr light/dark cycle (light from 6 a.m. to 6 p.m.). They were fed a normal pellet diet (GAFCO, Baghdad), and water was given *ad libitum*. All procedures complied with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in research, as well as local regulations and ethical considerations. All selected animals were examined before beginning of the study and were determined to be normal on ophthalmic and general examinations. Chronic ocular hypertension was induced by a single injection of α -chymotrypsin (BDH, Pool, England) into the posterior ocular chamber in animals anesthetized by an intramuscular injection of 35 mg/kg Ketamine (Imalgene 1000, Rhone Merieux) and 4 mg/kg Xylazine (Rompun 2%, Bayer).

Fifteen minutes before the injection, two drops of 50 μ L Pilocarpine hydrochloride were instilled. The anterior chamber was entered with a 25-gauge needle, near the limbus, and a 27-gauge needle was introduced directly into the posterior chamber through the pupil in order to inject 0.1 ml (450 EAU) of α -chymotrypsin (α -chymotrypsin 450 EAU, BDH, Pool, UK) into the posterior chamber of the right eye only. The left eye was not injected. The tip of the needle was swept across in order to homogeneously distribute the enzyme into the posterior chamber, and the needle remained in the posterior chamber for at least 1 min before being carefully removed to avoid any contact of the enzyme to the corneal endothelium. A daily ocular examination was performed during the following 2 weeks. For 4-5 days after α -chymotrypsin injection, one drop of Diclofenac Sodium (Jamjoom Pharma, KSA) and Chloramphenicol combined with Dexamethasone Disodium Phosphate (SDI, Iraq) were administered three times per day. In cases of severe ocular inflammation (which occurred in about 10% of the animals), the animals were not included in the study. One month after α -chymotrypsin injection, when an IOP higher than 25 mmHg in the right eye (as measured by transpalpebral and indentation based rebound tonometry) and no sign of inflammation are noticed. 36 rabbits among 60 injected with α -chymotrypsin were eligible for

inclusion in the experiments.

Study of Single Instillation

For evaluation of the drug activity following a single instillation, a 50 μ l (2 x 25 μ l) amount of a 0.75% SDH, 0.5 % Betaxolol, 0.25% and 2% Pilocarpine drops was applied topically to the α -chymotrypsin-treated right eye of three groups animals, each includes 12 albino rabbits; IOP was measured prior to drug instillation and then every hour for 7 hours after the treatment; the results were recorded as 5 change in IOP.

Study of Repeated Instillations

Repeated daily ocular applications were performed on the 3 groups of animals, each includes 12 albino rabbits. Each animal received two drops of 0.75% SDH, 0.5 % Betaxolol and 2% Pilocarpine drops in the α -chymotrypsin-treated right eye only. The treatment was applied two times a day: at 9:00 a.m. and at 5:00 p.m. from Day 1 to Day 12. During the course of the study, the IOP was measured daily for 5 days, before the beginning of the treatment (Day-5 to Day-1) and then every day (Day 1 to Day 12). To avoid errors due to the diurnal pressure variations, the IOP was always recorded three times a day: at 9:00 a.m. before the first instillation (t = 0), then at 12:00 a.m. (t = 3 hours) and at 4:00 p.m. before the second instillation (t = 7 hours). The results were expressed as % changes in IOP.

Statistical Analysis

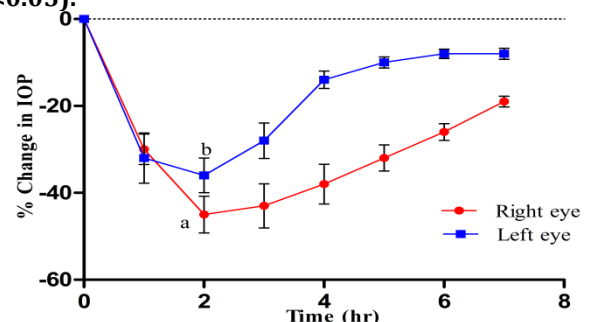
Results were presented as a mean value of IOP \pm S.D. Comparisons with baseline were made using Student's paired *t*-test, while a single-factor analysis of variance (ANOVA) was used to test the statistical differences between groups. In all experiments, comparison between treatments groups was performed by repeated measure analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test, unless otherwise specified. *P* values below 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of single corneal instillation of 0.75% SDH in α -chymotrypsin-induced glaucoma in right eye of rabbits

In figure 1, single instillation of 0.75% SDH drops in hypertensive right eyes of rabbits (IOP elevation induced by α -chymotrypsin) produced significantly greater decrease in IOP after 2 hrs compared to the effect produced in normotensive left eyes. The decrease in IOP was maintained at significantly greater level in the right eyes to the end of the follow up period (7 hrs) compared to that reported in the left eyes (-19% vs. -8%, *P*<0.05).

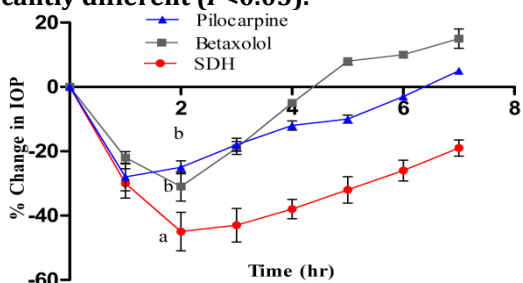
Figure 1. Effect of corneal instillation of single dose 0.75% SDH on right rabbit's eyes with elevated IOP and normotensive right eyes; values with non-identical letters (a,b) are significantly different (*P*<0.05).



In α -chymotrypsin-induced right eye hypertension, single corneal instillation of 0.75% SDH drops significantly decreases the elevated IOP after 1 hr (-30%), which was

significantly greater than that produced by 0.5% Betaxolol and equivalent to that produced by 2% Pilocarpine. After 2 hrs, SDH decreases IOP significantly greater than Betaxolol and Pilocarpine (-45%, -31% and -25%, respectively; $P<0.05$); this effect remains until the end of follow up period (7 hrs), where SDH maintains -19% decrease in IOP, while in both Betaxolol and Pilocarpine IOP was re-elevated by 15% and 5% respectively above the baseline values (Figure 2).

Figure 2. Effect of single corneal instillation of 0.75% SDH, 0.5% Betaxolol and 2% Pilocarpine eye drops on IOP in rabbit's model of right eye glaucoma; values with non-identical letters (a,b,c) are considered significantly different ($P<0.05$).



Effect of long-term corneal instillation of 0.75% SDH drops in α -chymotrypsin-induced glaucoma in right eye of rabbits

The mean effect of repeated corneal instillation of 0.75% SDH, 0.5% Betaxolol and 2% Pilocarpine eye drops for 12 consecutive days are summarized in figures 3, 4, 5, and 6. The obtained results showed that a gradual daily decrease of IOP at the third hr post-instillation occurred during treatment. The mean of % IOP decrease in SDH-treated group was significantly greater than that reported in both Betaxolol and Pilocarpine, which produce comparable effect in this respect. Figures 3 and 4 clearly showed the daily variations in IOP lowering activity, which is more prominent in SDH-treated group of rabbits compared to comparators (Betaxolol and Pilocarpine), although the % decrease in IOP was significantly greater when reported both after 3 and 7 hrs.

Figure 3. Comparison between long-term corneal instillation of 0.75% SDH, 0.5% Betaxolol and 2% Pilocarpine eye drops on IOP of rabbit's model of glaucoma after 3 hours.

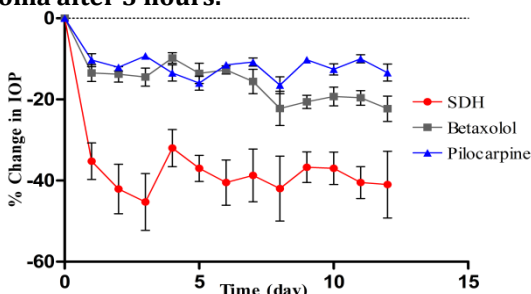


Figure 4. Comparison between long-term corneal instillation of 0.75% SDH, 0.5% Betaxolol and 2% Pilocarpine eye drops on IOP of rabbit's model of glaucoma after 7 hours.

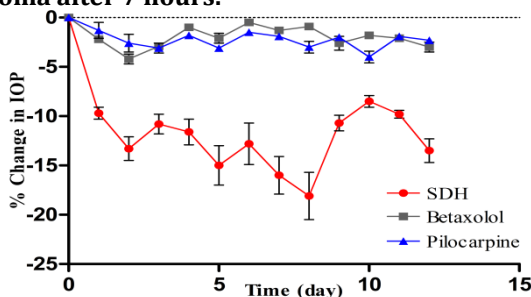


Figure 5. Comparison between the means of % IOP changes due to long-term corneal instillation of 0.75% SDH, 0.5% Betaxolol and 2% Pilocarpine eye drops in α -chymotrypsin-induced ocular hypertension in rabbits after 3 hrs; values with non-identical letters (a,b,c) are significantly different ($P<0.05$).

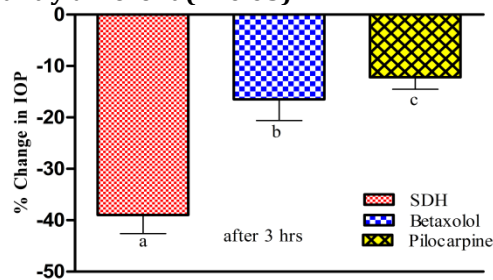
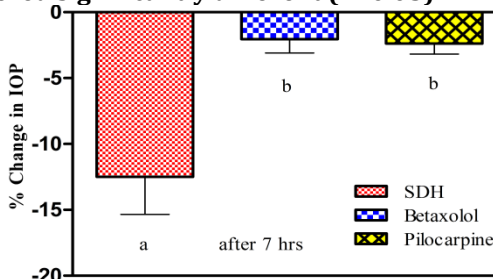


Figure 6. Comparison between the means of % IOP changes due to long-term corneal instillation of 0.75% SDH, 0.5% Betaxolol and 2% Pilocarpine eye drops in α -chymotrypsin-induced ocular hypertension in rabbits after 7 hrs; values with non-identical letters (a,b) are considered significantly different ($P<0.05$).



The ability of Silibinin to inhibit many isoforms of cAMP-PDE enzyme is very well characterized¹⁰, and accordingly this study was designed to evaluate the efficacy of SDH drops in lowering IOP in normotensive rabbits and in α -chymotrypsin-induced ocular hypertension, for the aim of introducing new antiglaucomatous agent with new pharmacological approach of treatment. Koch *et al* described Silibinin to possess strong inhibitory action on cAMP-PDE in beef heart.¹⁷ Although the action of Silibinin on eye's PDE has not been studied; but if such an action would be expected there, the ocular hypotensive effect of this flavonoid can be explained accordingly. Meanwhile, accumulation of intracellular cAMP is likely to underlay the mechanism of lowering IOP by other agents such as epinephrine and Forskolin.^{6,18,19} Do *et al* have demonstrated that cAMP inhibits net transepithelial chloride secretion into the posterior chamber, and directly activates Cl⁻ channels of native PE cells, which may contribute to facilitation of chloride ion reabsorption into the ciliary stroma; they suggested that these effects may provide potential mechanisms for the reduction and modulation of net aqueous humor secretion.^{20,21} Although inhibition of cAMP-phosphodiesterase is proposed as a suspected mechanism for the action of SDH on IOP¹⁰, interference with the cholinergic influence in this respect was evaluated. In the present study, the effect of SDH on IOP was higher than that produced by Pilocarpine, and their combination results in an additive effect. Muscarinic agonists, including Pilocarpine, lower IOP through enhancing AH outflow due to contraction of the iris sphincter.²² According to the reported mechanisms of action of SDH, targeting AH formation and interference with ion transport can be suggested as possible mechanisms.²³ Consequently, the mechanisms through which Pilocarpine and SDH produce their effects can be utilized for explaining the additive effect reported when both of them are used at the same time. We previously

confirm the idea that SDH lowers IOP through a mechanism not related to the cholinergic system, through evaluating the interaction of SDH with anticholinergic agents like Cyclopentolate.¹³ Although there is no practical evidence on elevation of IOP in rabbits due to instillation of Cyclopentolate, the previously reported data demonstrated such effect, which can be attributed to the abnormal sensitivity of the locally bred strain of rabbits to the effect of Cyclopentolate. In the present work, the rise in IOP produced by instillation of Cyclopentolate was effectively reversed by Silibinin (unpublished data); meanwhile, the IOP lowering effect of SDH was not affected by post-instillation of Cyclopentolate. Based on these data, one can postulate that SDH interferes with IOP regulation through reduction of AH inflow. Taken together with the data obtained in previous study¹² about the effect of SDH on IOP recovery rate and its contra-lateral effect, one can suggest the interference with AH inflow as a mechanism involved in Pilocarpine-SDH and Cyclopentolate-SDH interactions. The IOP lowering effect of SDH is thought to occur via reduction of AH formation, and the site of action has been postulated to be the ciliary epithelium; this is based on previous data reported in our laboratory that revealed delayed recovery time following intravenous infusion of 20% NaCl and a profound contralateral effect on untreated eyes.¹²

The present study demonstrated that when compared with Betaxolol, SDH was found more effective in lowering IOP. It appears that neither pre- nor post-instillation of each one of them improves significantly the IOP-lowering effect produced by any one of them alone (unpublished data). Pre-instillation effect of SDH appears to completely abolish that of Betaxolol; however, the higher magnitude of reduction in IOP already produced by pre-instillation of SDH was due to the action of SDH alone. These effects are very interesting in that the potent action of SDH might

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mask that of Betaxolol especially when given 30 min before, and this might explain the predominance of SDH action over that of Betaxolol. However, SDH did not augment the effect of Betaxolol when administered latter suggesting interference with its action by previous instillation of Betaxolol. This conclusion can be accepted pharmacodynamically since β -blockers are known to initiate decrease in cAMP levels required for the action SDH (as PDE-inhibitor) and the only effect shown might be attributed to betaxolol alone. Although the effect of SDH on the ocular phosphodiesterase (PDE) has not been studied, a study on beef heart PDE revealed that SDH was more potent as PDE-inhibitor than Theophylline and Papaverine in this regard.¹⁴ From these findings one can suggest that the strong ocular hypotensive effect produced by SDH might be attributed to the inhibition of PDE and the resultant accumulation of cAMP inhibits $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transporter in ciliary epithelium as well as in trabecular meshwork cells. Both effects on inflow and outflow of aqueous humor dynamics could be the possible mechanisms through which SDH produces this effect and became a new drug candidate for the reduction of elevated IOP.

CONCLUSION

In conclusion, the novel formula of SDH eye drops was effective in management of glaucoma by decreasing IOP, probably through interfering with both inflow and outflow of aqueous humor dynamics, and it may be a new drug candidate for the reduction of elevated IOP.

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