

Effects of silibinin hemisuccinate on the intraocular pressure in normotensive rabbits

Haider M. Mohammed, BSc, MSc, Munaf H. Abdulrazzaq, BSc, MSc, Saad A. Hussain, MSc, PhD.

ABSTRACT

Objective: To evaluate the effects of silibinin hemisuccinate on the normal intraocular pressure (IOP) in rabbits.

Methods: This study took place in the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad during the period from January to June 2005. Twenty-five New Zealand white rabbits weighing 1.5-2.5 kg were used in this study. The effects of corneal instillation of various concentrations of silibinin hemisuccinate (0.5%, 0.75% and 1%) dissolved in arachis oil, on the normal intraocular pressure in rabbits were evaluated using indentation tonometry; in addition to the possible modulation of normal IOP-recovery time after intravenous infusion of 20% sodium chloride solution.

Results: The results showed that within 30 minutes of application, silibinin in various concentrations significantly reduces IOP in comparison to baseline values ($p < 0.05$), with greater reduction being achieved with 0.75%. The effect of IOP reduction lasts 2-3 hours and proportionate to the concentration used. Moreover, remarkable delay in IOP recovery was observed after instillation of silibinin compared with the vehicle treated (arachis oil) animals, indicating interference with aqueous humor formation.

Conclusion: The results obtained in this study provide experimental evidences for the effectiveness of silibinin in the reduction of IOP and possible modulation of its regulatory mechanisms.

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From the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq.

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Address correspondence and reprint request to: Dr. Saad A. Hussain, Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq, Tel. +964 (25) 532418. E-mail: saad_alzaidi@yahoo.com

The intraocular pressure (IOP) reflects a balance between the rate of formation and exit of aqueous humor. Glaucoma, a major leading cause of blindness, is usually associated with elevated IOP. Pharmacological treatment of ocular hypertension is now largely directed toward reducing aqueous humor formation and/or increasing its drainage using a wide range of agents, including carbonic anhydrase inhibitors, alpha-adrenergic and cholinergic agonists,¹ prostaglandin analogs,² and beta-adrenergic blockers.³ The precise mechanisms underlying aqueous humor secretion into the eye are uncertain, but the release of Cl⁻ through the basolateral channels of the non-pigmented epithelial (NPE) cells is thought to be rate limiting.^{4,5} Thus, agents that can alter this chloride conductance either directly or indirectly will alter aqueous humor production. Many recent observations suggest an important role for both intra- and extracellular ATP and adenosine in the regulation of ciliary epithelial chloride conductance and formation of aqueous humor.⁶ Purinergic receptors for adenosine and ATP are expressed by both NPE and pigmented epithelial (PE) cells of the ciliary epithelial layers.⁷ Direct stimulation of alpha-adrenergic receptors via the interaction with G_s protein increases cAMP production,⁸ while forskolin acts as a direct stimulator of adenylyl cyclase, increases endogenous production of cAMP with consequent decrease in aqueous humor inflow.⁹ There is another possibility for elevating cAMP levels through the inhibition of cAMP-phosphodiesterase; silymarin, the active principle of milk thistle (*Silybum marianum*) is found to be a very potent inhibitor of cAMP breakdown by phosphodiesterase, even more potent than theophylline and papaverine in this respect.¹⁰ The present study was designed to evaluate the IOP lowering effect of silibinin hemisuccinate in normotensive rabbits.

Methods. The study took place in the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad during the period from January

to June 2005. Twenty-five New Zealand white rabbits weighing 1.5-2.5 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 a standard diet and given water ad libitum. Silibinin hemisuccinate in pure form and all other chemicals were supplied by the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad. Rabbits were allocated into 5 groups, 5 rabbits in each one, for studying the effect of 3 concentrations of silibinin hemisuccinate (0.5%, 0.75% and 1%) and the vehicle arachis oil, instilled into both eyes, on the normal IOP and the effect of the most effective concentration reported for silibinin on the IOP-recovery time after intravenous infusion of 20% sodium chloride, as an experimental marker for the possible interference with the rate of aqueous inflow.

Measurement of intraocular pressure. Indentation tonometry, using Schiøtz tonometer, was followed in this study for measuring IOP before and after instillation of silibinin or arachis oil as a vehicle used for preparing drug solutions. Thirty minutes before starting any application, the cornea was anesthetized with 0.5% tetracaine HCl, and baseline IOP was measured. After a single topical instillation of one drop of silibinin hemisuccinate (0.5%, 0.75%, 1%) or arachis oil, IOP was measured every 30 minutes for 3 hours.¹¹ After each measurement, eyes were washed with normal saline and the instrument was cleaned with diethyl ether. All measurements of IOP were performed by an experienced technician who was masked to the treatment allocation of that particular animal, and performed during a fixed time of the day (from 10:00 am to 3:00 pm) to exclude the effect of circadian changes in IOP. For studying the effect of silibinin on IOP-recovery time, through the possibility of interference with aqueous humor formation, 5 rabbits (control group) were used for measurement of the baseline IOP in both eyes, 60 minutes prior to infusion of hypertonic NaCl (20%) at a rate of 1 ml/min for 10 minutes and during 5, 10, 15 and 30 minutes until IOP recovered to the pre-infusion value. After one week, the same group of rabbits (used as control) was utilized to evaluate the effect of 0.75% silibinin on the IOP recovery time as indicated previously. The IOP-recovery rate was expressed as the slope of the IOP recovery line calculated through the points 15 and 120 min after NaCl infusion. The differences in the slope and percent changes between controls and silibinin-treated animals were analyzed using unpaired Student's t-test. Shallow slope, compared to the control, indicates prolonged IOP-recovery and reduced aqueous humor inflow.¹²

All results are presented as mean \pm SE. Comparisons with baseline values were made using Student's paired t-test, while a single-factor analysis of variance (ANOVA) for repeated measurements was used to test the statistical significance of differences between groups. P values less than 0.05 were considered significant.

Results. In normotensive rabbits, instillation of arachis oil did not show any significant changes in IOP along the entire period of experiment, while ocular instillation of silibinin hemisuccinate in 3 concentrations (0.5%, 0.75% and 1.0%) produced a decrease in IOP, maintained for 2-3 hr and remain significant compared to baseline value at all time points (**Table 1 & Figure 1**). Concentrations lower than 0.5% did not change IOP (data not shown), while 1.0% silibinin showed lower effect compared to 0.5% and 0.75% solutions. Maximum decrease in IOP was achieved after one hour of instillation of each concentration of silibinin, and the largest effect was reported with 0.75% solution (39%) compared to 0.5% and 1% solutions (28% and 23%, $p < 0.05$). Furthermore, 0.5% silibinin solution produced longer duration of IOP lowering action, while 1% solution produced the shorter one (**Figure 1**). For this reason, 0.75% silibinin solution was selected for investigating the effect on normal IOP-recovery. One hour after corneal instillation of one drop of the drug vehicle (arachis oil), intravenous infusion of 20% NaCl at a rate of 1 ml/min for 10 min resulted in rapid fall in IOP, with a peak reduction achieved after 15 min (74%, $p < 0.05$); then IOP rises gradually and returned to its baseline value within 2.5 hr (**Table 2 & Figure 2**). Instillation of 0.75% silibinin, 60 min prior to NaCl infusion, resulted in higher reduction in IOP compared to that reported in controls, with peak reduction obtained 30 min after instillation (97%, $p < 0.05$), followed by gradual and slower increase in IOP. At the period of 2.5 hours, IOP was still significantly lower (44%) than that achieved in controls (5%, $p < 0.05$). Furthermore, when the values of IOP recovery time are plotted versus time, from the point 15 min after starting NaCl infusion up to 120 min, a shallow slope obtained with the use of 0.75% silibinin compared to that reported during normal recovery (**Figure 3**).

Discussion. Cyclicadenine dinucleotide phosphate (cAMP) mediates many biological processes through its ability to stimulate cyclic nucleotide-dependent protein kinase, which in turn phosphorylates cellular protein substrates and initiates specific responses. This regulatory activity of cAMP was terminated by the action of the enzyme cyclic nucleotide phosphodiesterase (PDE). Cyclic nucleotides are involved in the regulation of many cellular processes in the eye, including the modulation

Table 1 - Effects of different concentrations of silibinin hemisuccinate oily solution on the intraocular pressure of normotensive rabbits (n=10).

Time (hr)	Intraocular pressure (mm Hg)			
	Vehicle (Arachis oil)	Silibinin hemisuccinate solution		
		0.50%	0.75%	1.0%
Baseline	32.3 ± 0.72	31.6 ± 0.65	28.6 ± 0.60	29.6 ± 0.44
0.5	32.3 ± 0.72	26.7 ± 1.07*	22.1 ± 0.73*	25.6 ± 0.54*
1.0	31.9 ± 1.04	22.9 ± 0.73*	17.6 ± 0.89*	22.7 ± 0.95*
1.5	31.9 ± 1.10	23.7 ± 0.80*	19.1 ± 1.17*	23.6 ± 1.20*
2.0	32.3 ± 1.17	24.6 ± 1.55*	25.3 ± 1.27*	26.7 ± 0.60*
2.5	32.3 ± 1.17	27.3 ± 0.92*	27.5 ± 0.92*	28.9 ± 0.54*
3.0	32.3 ± 0.91	29.2 ± 0.92*	28.6 ± 0.60	29.7 ± 0.44

Data were expressed as mean ± SE.
*significantly different compared to baseline value (p<0.05).

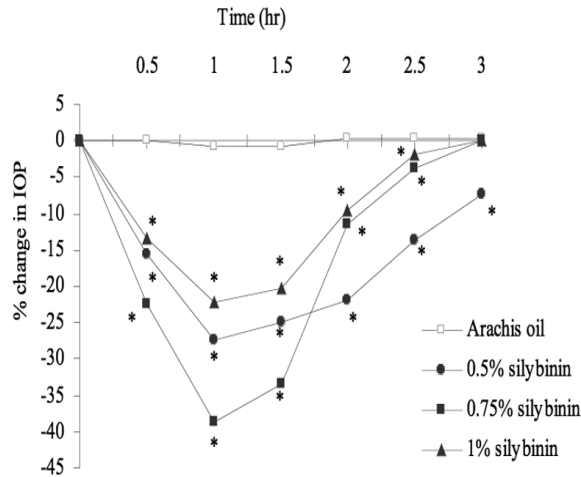


Figure 1 - Effect of topically applied silibinin hemisuccinate (0.5%, 0.75% and 1.0% in arachis oil) on intraocular pressure (IOP) in normotensive rabbits. Results presented as mean percent change in IOP; number of eyes = 10, *significantly different compared to baseline value (p<0.05).

Table 2 - Effect 0.75% silibinin solution on the intraocular pressure (IOP) recovery rate after intravenous infusion of 20% sodium chloride solution in normotensive rabbits (n=10).

Time (min)	Intraocular pressure (mm Hg)	
	Normal IOP recovery	IOP recovery after instillation of 0.75% Silibinin
Baseline	31.6 ± 0.63	31.8 ± 0.57
10	11.3 ± 0.63*	2.5 ± 0.16*
15	8.1 ± 0.92*	1.7 ± 0.16*
30	8.1 ± 1.08*	1.0 ± 0.6*
60	13.6 ± 1.42*	5.9 ± 0.32*
90	22.0 ± 1.08*	4.9 ± 0.25*
120	28.7 ± 1.46*	11.1 ± 0.76*
150	29.7 ± 0.89*	17.6 ± 0.85*

Data presented as mean ± SE.
*significantly different compared to baseline value (p<0.05).

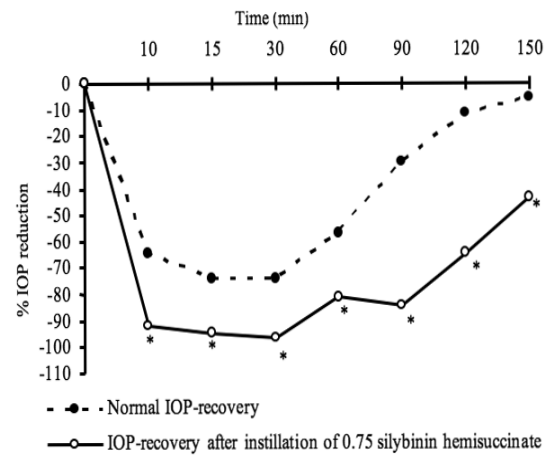


Figure 2 - Effect of topically applied silibinin hemisuccinate (0.75% in arachis oil) on intraocular pressure (IOP) -recovery time in normotensive rabbits. Results presented as mean percent change in IOP; number of eyes =10. All points in normal IOP-recovery and after application of 0.75% silibinin are significantly different with respect to baseline *p<0.05.

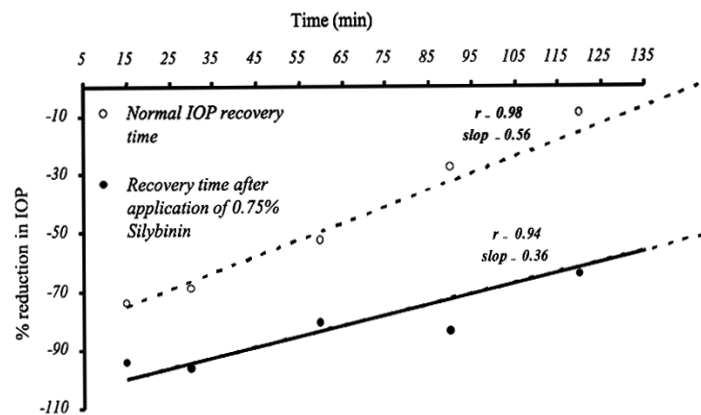


Figure 3 - Effect of 0.75% silibinin hemisuccinate on the intraocular pressure (IOP)-recovery time. Shallow slope indicates prolonged (delayed) IOP-recovery.

of aqueous humor dynamics.¹³ The ability of silibinin to inhibit many isoforms of cAMP-PDE enzyme is very well characterized,¹⁴ and accordingly this study was designed to evaluate the efficacy of silibinin in lowering IOP in normotensive rabbits, for the aim of introducing new antiglaucomatous agent with new pharmacological approach of treatment.

The data presented in **Table 1** and **Figure 1** clearly showed that topical application of various doses of silibinin resulted in significant decrease in IOP, with maximum effect obtained with 0.75% dose. Distinct profile of PDE isozymes activity have been reported in cultured cells derived from bovine PE and human NPE, as well as corresponding differences in the effect of selective PDE inhibitors in the accumulation of cAMP and cGMP.¹⁵ It has been reported that PDE¹ is the mostly abundant isoform of cAMP-PDEs in the NPE cells,¹⁶ and its blockade by silibinin there lead to accumulation of cAMP with consequent lowering of IOP. However, the decrease in the IOP lowering effect of 1% silibinin solution (**Figure 1**) could be attributed to the inhibition and/or modulation of the activities of other isoforms of PDE, including cGMP-PDEs in the PE cells. Therefore, alteration of either cAMP or cGMP levels modulates the production of aqueous humor by the ciliary epithelial cells of the eye with consequent effect of IOP. Koch et al¹⁰ described silibinin to possess strong inhibitory action on cAMP-PDE in beef heart.¹⁰ 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.¹⁷⁻¹⁹ Do et al²⁰ 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 Cl⁻-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} Interestingly, the mechanism through which prostaglandin analogues enhance uveoscleral outflow and hence reduce IOP, depends on the accumulation of cAMP as a main messenger linking several events following administration of these agents, like the formation of intramuscular spaces and stimulation of matrix metalloproteinase.²² These agents have been reported to produce 50% reduction in IOP, an effect being comparable to that produced by silibinin in the present study. This may be added to the idea that enhancement of uveoscleral outflow, brought about by accumulation of cAMP, can be used to explain such high reduction in IOP reported in the present study.

The effect of various compounds on aqueous humor inflow into the posterior chamber of the rabbit's eye can be used to determine the mechanism of action, and compare the potency of IOP lowering agents.¹² In the present study, intravenous infusion of 20% NaCl solution produces rapid and marked fall in IOP; the peak reduction in IOP occurs 15 min after starting infusion and recovers within approximately 150 min. Instillation of 0.75% silibinin 60 min prior to NaCl infusion significantly delayed recovery time to more than 150 min (**Table 2 & Figure 2**). Infusion of hypertonic NaCl causes decrease in cell volume and shrinkage of ciliary epithelial cells. Normally, these cells return to their original volume by operating regulatory volume increase (RVI) mechanism, which require 120 min to restore original volume.²³ This mechanism was blocked by cAMP elevation, with consequent blockade of Na⁺-K⁺-2Cl⁻ co-transporter in the ciliary epithelial cells.^{20,24} As silibinin was shown to prolong IOP recovery time, one can suggest the interference with RVI mechanism to be the likely pathway involved in this respect. Moreover, the increased cAMP level because of PDE inhibition may be the main event linking the effect of silibinin to the lowering of elevated IOP. Taken together, these findings supported the idea that silibinin exerts powerful inhibition on aqueous humor inflow as a possible mechanism for IOP lowering effect.

In conclusion, silibinin, utilizing the mechanism of PDE-inhibition and/or other mechanisms, produced dose dependent reduction in IOP when topically instilled on the eyes of the rabbit.

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