

## Effect of Silibinin in Lowering the Intraocular Pressure in Normotensive Rabbits: Interaction with Betaxolol

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### Abstract

Previous reports demonstrated the effectiveness of silibinin hemisuccinate as a potential intraocular pressure-lowering agent. The exact mechanism by which silibinin exerted this effect has not yet been documented, but might suggested to interfere with aqueous humor formation. The present study was designed to evaluate the comparative efficacy of silibinin as IOP lowering agent to that of betaxolol in normotensive rabbits, and the interaction of silibinin with betaxolol as a way for investigating the possible mechanism of action of silibinin in this respect. The effects of instillation of 0.75% silibinin solution and 0.5% betaxolol eye drops in the eyes of normotensive rabbits were evaluated using indentation tonometry. The results showed that 0.75% solution of silibinin was more potent than betaxolol (0.5%) in lowering IOP in normotensive rabbits. Furthermore, the effect of pre- and post-instillation of silibinin-betaxolol combination showed a characteristic antagonistic feature. In conclusion, silibinin appears to be more potent than betaxolol in lowering IOP in normotensive rabbits; the pre- and post-instillation of silibinin provide experimental evidence for the possible antagonistic effect of betaxolol with the IOP-lowering effect of silibinin.

**Key words:** silibinin, betaxolol, cAMP, PDE-inhibitors

### الخلاصة

ثبتت من دراسة سابقة فعالية مادة السيليبينين في خفض الضغط الداخلي للعين، إلا ان ميكانيكية مثل هذا التأثير لم تعرف بعد على الرغم من احتمال تداخلها مع تكوين السائل الداخلي للعين. تم تصميم هذه الدراسة لمقارنة فعالية السيليبينين في خفض الضغط الداخلي للعين مقارنة بمادة البيتاكسولول في الارانب، وكذلك دراسة التداخل بين البيتاكسولول والسيليبينين عند استخدامهما معا كوسيلة لتكوين فكرة حول ميكانيكية عمل السيليبينين. تمت دراسة تأثير الاستخدام الموضعي لمحلول 0.75% سيليبينين و 0.5% بيتاكسولول قطرات في عيون أرانب بيضاء ذات ضغط داخلي طبيعي باستخدام تقنية indentation tonometry. أظهرت النتائج فعالية السيليبينين في خفض الضغط الداخلي للعين بشكل افضل من ذلك الذي يحدثه البيتاكسولول، كما ان استخدام السيليبينين قبل وبعد البيتاكسولول اظهر تضادا واضحا في التأثير. يمكن الاستنتاج بان الاستخدام الموضعي للسيليبينين في العين يؤدي الى خفض الضغط الداخلي للعين اكثر من البيتاكسولول، كما ان التداخل السلبي مع البيتاكسولول يمكن ان يعطي فكرة أولية عن ميكانيكية عمل هذا المركب.

### Introduction

Although glaucoma is no longer defined as elevated intraocular pressure (IOP) but rather a condition comprises characteristic optic nerve head and visual field abnormalities <sup>(1)</sup>, lowering IOP is still the major strategy in slowing down glaucomatous damage to the inner structures of the eye and visual field <sup>(2)</sup>. 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 <sup>(3)</sup>. The ciliary epithelium has  $\alpha$ - and  $\beta$ -adrenergic receptors. Stimulation of  $\alpha$ -receptors or inhibition of  $\beta$ -receptors was thought to reduce AH formation <sup>(4)</sup>. Topical instillation of epinephrine decreases the rate of AH formation, an effect thought to be mediated by  $\beta$ -receptor induced increase in cAMP in the ciliary epithelium <sup>(5)</sup>. The participation of cAMP in this effect has been supported by

finding that activators of adenylyclase (cholera toxin and forskolin) decrease AH formation and hence IOP in experimental animals and human <sup>(6)</sup>. Targeting of this Cl<sup>-</sup> transport system is thought to be the newer proposed mechanism for the lowering of IOP by the oldest antiglaucomatus drug, Timolol <sup>(7)</sup>. 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  $\beta$ -blockers in the reduction of AH formation is now suggested to involve cAMP-independent mechanism <sup>(7)</sup>. Furthermore, timolol was shown to reduce epinephrine-induced increase in uveoscleral outflow when the two drugs applied concurrently <sup>(8)</sup>. Inhibition of phosphodiesterase (PDE) by flavonoids has been previously described <sup>(9,10)</sup>; silib

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powerful antioxidant flavonoid <sup>(11)</sup> has been shown to reduce IOP in normotensive rabbits when used alone in different concentrations, with greater effect achieved with 0.75% dose <sup>(12)</sup>. 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, silibinin was shown to inhibit cAMP-phosphodiesterase enzyme more potent than theophylline or papaverine <sup>(13)</sup>. This study was conducted to evaluate the possible interaction of silibinin with the IOP-lowering effect of the  $\beta$ -adrenergic blocker betaxolol in normotensive rabbits.

### Materials and Methods

Twenty 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 standard diet and given water *ad libitum*. Silibinin hemisuccinate in pure form was a gift from Tolbiac S.R.L. (Argentina) and all other chemicals were supplied by the Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad. Silibinin hemisuccinate was dissolved in arachis oil (as a vehicle) and used as freshly prepared (0.75%) solution. Betaxolol 0.5% drops (Alcon, Cham, Switzerland) were used as commercial eye drop formula. Rabbits were allocated into four groups (5 animals each) for studying the effect of topically instilled silibinin hemisuccinate, betaxolol, the effect of topical silibinin instilled 30 min prior to betaxolol, and the effect of topical betaxolol instilled 30 min prior to instillation of silibinin. Measurement of IOP: Indentation tonometry using Schiøtz tonometer was utilized in this study for measuring IOP before and after application of drugs or vehicle. Thirty min before starting any application, the cornea was anesthetized with 0.5% tetracaine, and baseline IOP was measured using Schiøtz tonometer. After topical instillation of 1 drop of silibinin (0.75%) and betaxolol (0.5%), measurement of IOP was performed every 30 min for 3 hr <sup>(14)</sup>. After each measurement, eyes were washed with normal saline and the instrument was cleaned with diethyl ether. All experiments were conducted by trained subject who is completely unaware about the type of treatment followed, 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 pre-instillation effect of betaxolol, baseline IOP was recorded after the instillation of betaxolol, and then one drop of silibinin solution was instilled after 30 min into both eyes. IOP was measured every 30 min for 3 hr. The same later approach was followed to evaluate the effect of pre-instillation of silibinin on that produced by betaxolol. Results were presented as a mean value of IOP  $\pm$  SD. 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. *P* values less than 0.05 were considered significant.

### Results

#### *Effects of 0.75% silibinin and 1% betaxolol:*

In normotensive rabbits, ocular instillation of 0.75% silibinin decreases IOP for 2.5 hr compared to baseline value and remains significantly different at all measured time points (Table 1). The maximum decrease in IOP was achieved after 1 hr of instillation of silibinin (38.56%) compared to baseline value ( $P < 0.05$ ). Application of 1 drop betaxolol (0.5%) eye drop resulted in significant decrease in IOP, with maximum reduction achieved after 1hr (22.08%); then decreased with time and became (2.05%) after 3.0 hr, which found non-significantly different compared to baseline (Table 1).

#### *Effects of pre-treatment with 0.75% silibinin*

Corneal instillation of 0.5% betaxolol eye drops produced highly significant reduction in IOP when preceded (30 min) by instillation of 0.75% silibinin (38.35%-3.9%,  $P < 0.05$ ) compared to the effect produced by betaxolol alone (22.08%-6.61%,  $P < 0.05$ ) (Table 1, Figure 1). The higher magnitude of reduction in IOP was achieved during the first 30 min of instillation of 0.5% betaxolol to silibinin-pretreated eyes (38.35%,  $P < 0.05$ ) compared to 10.78% produced by betaxolol alone. The reduction in IOP continued to be significantly high during the next 30 min interval (27.76%,  $P < 0.05$ ) compared to 22.08% produced by 0.5% betaxolol alone. However, the reduction in IOP after this period seems to be non-significant (17.71%, 10.52%, 3.94%,  $P > 0.05$ ) compared to 19.49%, 20.87% and 6.61% produced by 0.5% betaxolol alone at the intervals 1.5, 2.0 and 2.5 hr ( $P > 0.05$ ). The reduction in IOP was significantly different from that produced by 0.75% silibinin alone for the intervals 0.5, 1.0 and 1.5 hr ( $P < 0.05$ ), while found non-significant for the rest of time ( $P > 0.05$ ) (Table, Figure 1).

**Effects post-treatment with silibinin**

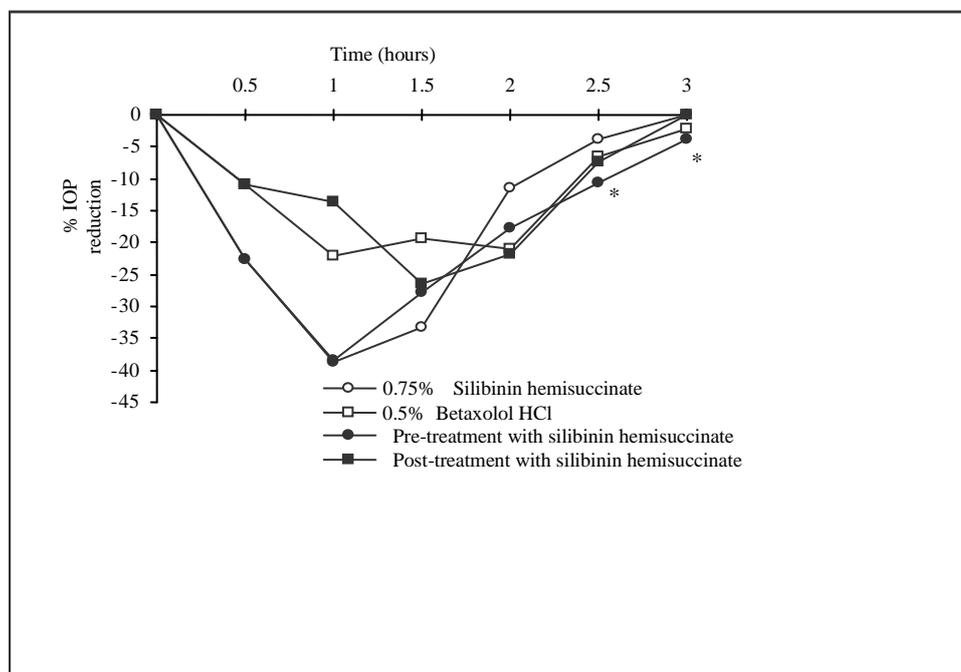
The results presented in table 1 and figure 1 showed that the ocular hypotensive effect of 0.5% betaxolol was slightly changed following the addition of silibinin, and during the first 30 min of application, 0.75% silibinin resulted in

13.61% reduction in IOP compared to 22.08% produced by betaxolol alone ( $P>0.05$ ). Although the IOP was reduced following the next 30 min (26.52%), it was found comparable to that produced by betaxolol alone ( $P>0.05$ ).

**Table 1. Effects of instillation of silibinin 0.75% and betaxolol 0.5% on the intraocular pressure (IOP) in normotensive rabbits.**

Intraocular Pressure (mmHg)							
Group	Baseline	0.5hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	3.0 hr
Silibinin 0.75%	28.6 ± 1.9	22.1 ± 0.6* <sup>a</sup>	17.5 ± 2.8* <sup>a</sup>	19.1 ± 3.7* <sup>a</sup>	25.3 ± 4.0* <sup>a</sup>	27.5 ± 2.9* <sup>a</sup>	28.6 ± 1.9 <sup>a</sup>
Betaxolol 0.5%	31.5 ± 2.3	28.1 ± 3.1* <sup>b</sup>	24.5 ± 3.9* <sup>b</sup>	25.4 ± 3.1* <sup>b</sup>	24.9 ± 2.3* <sup>a</sup>	29.4 ± 2.4* <sup>a</sup>	30.8 ± 2.0 <sup>a</sup>
Silibinin pre-treatment	31.7 ± 2.5	24.6 ± 2.2* <sup>c</sup>	19.7 ± 2.3* <sup>a</sup>	22.9 ± 2.7* <sup>a</sup>	26.1 ± 2.2* <sup>a</sup>	28.4 ± 2.5* <sup>a</sup>	30.5 ± 2.4 <sup>a</sup>
Silibinin post-treatment	31.7 ± 2.8	28.1 ± 3.4* <sup>b</sup>	27.2 ± 3.6* <sup>b</sup>	23.1 ± 2.6* <sup>b</sup>	24.7 ± 3.1* <sup>a</sup>	29.2 ± 2.6* <sup>a</sup>	31.5 ± 2.7 <sup>a</sup>

Values are expressed as mean ± SD; number of eyes in each group = 10; \*  $P<0.05$  with respect to baseline value; values with non-identical superscripts (a,b,c) among different groups are considered significantly different ( $p<0.05$ ).



**Figure 1. Effects of pre- and post- treatment with 0.75% silibinin hemisuccinate on IOP-lowering effect of 0.5% betaxolol in normotensive rabbits; results are presented as mean of % reduction; number of eyes in each group = 10; \* $P<0.05$  with respect to 0.75% silibinin hemisuccinate alone.**

## Discussion

In the present study, topical instillation of silybinin was shown to strongly lower IOP in normotensive rabbits, an effect that appears significantly greater than that produced by 0.5% betaxolol (Table1). Betaxolol binds to  $\beta$ -adrenergic receptors of the ciliary processes with high affinity<sup>(15)</sup>. Since agonists to all  $\beta$ -adrenergic receptors ( $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ ) stimulate adenylcyclase via interaction with Gs-protein to increase cAMP production, betaxolol was thought to lower IOP by reducing the intracellular concentrations of cAMP<sup>(16)</sup>. However, it has long been unclear whether the putative reduction in cAMP itself causes the reduction in IOP, an observation reported by Liu *et al* (1981) who demonstrated that D-timolol, another  $\beta$ -blocker might be as effective as L-timolol in decreasing aqueous flow<sup>(17)</sup>, despite stereospecificity of the  $\beta$ -adrenergic receptors for the L-isomers<sup>(18)</sup>. Meanwhile, if betaxolol reduces aqueous humor formation by blocking  $\beta$ -adrenergic-mediated increase of cAMP production, one would expect cAMP itself to increase inflow. However, cAMP certainly does not markedly increase aqueous inflow. Accordingly, Caprioli *et al* (1984) reported a decrease in inflow following administration of forskolin, which stimulates endogenous production of cAMP<sup>(6)</sup>. However, the foregoing considerations do not preclude the possibility that betaxolol reduces secretion of aqueous humor exclusively through its action as a nonselective  $\beta$ -adrenergic antagonist, but have raised doubts about that hypothesis. A conflicting result have recently been reported by McLaughlin *et al* (2001) who demonstrated that application of cAMP did not reverse timolol's effects; and that timolol and levobunolol produced cAMP-independent inhibition of the regulatory volume increase (RVI) in ciliary cells and increased intracellular  $Ca^{2+}$  and pH; they suggested that inhibition of  $Cl/HCO_3^-$  exchange mediates timolol's inhibition of aqueous humor formation as an alternative mechanism for the reduction of aqueous inflow and then IOP<sup>(7)</sup>. The IOP lowering effect of silibinin 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<sup>(12)</sup>. The present study demonstrated that when compared with betaxolol, silibinin 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. Pre-instillation effect of silibinin appears to completely abolish that of betaxolol; however, the higher magnitude of reduction in IOP already produced by pre-instillation of silibinin (figure 1) might be due to the action of silibinin alone. These effects are very interesting in that the potent action of silibinin might mask that of betaxolol especially when given 30 min before, and this might explain the predominance of silibinin action over that of betaxolol (Figure 1). However, silibinin 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  $\beta$ -blockers are known to initiate a decrease in cAMP levels required for the action silibinin (as PDE-inhibitor) and the only effect shown in figure 1 might be attributed to betaxolol alone. The mechanism through which betaxolol reduced IOP is now difficult to correlate with its inhibitory effect on cAMP. Isoproterenol, which stimulate cAMP production, has been reported to increase aqueous humor inflow<sup>(19)</sup>. In contrast, forskolin, which also stimulate cAMP formation, has been found to reduce inflow<sup>(20)</sup>, and isoproterenol itself has reported to reduce IOP in water-loaded rabbits<sup>(21)</sup>. Since silibinin has been proved to inhibit PDE<sup>(9)</sup> and to produce higher magnitude of reduction in IOP (37.84%) compared to that of betaxolol (22.0%), one could suggest that increasing intracellular cAMP could be the major event through which silibinin reduces IOP. Involvement of cAMP as a target in the events of lowering IOP was clear in many studies that involve application of forskolin (an adenylcyclase activator) via either topical or systemic route<sup>(22)</sup>. Forskolin reduced net aqueous humor inflow in rabbits, and increased ciliary blood flow through activation of adenylcyclase in ciliary epithelium; this action was not blocked by timolol<sup>(6)</sup>. This is quite important to explain why betaxolol blocks the effect of silibinin (a PDE-inhibitor).  $\beta$ -blockers did not block the action of forskolin because the latter was still capable to stimulate synthesis of cAMP, while silibinin requires the presence of already synthesized cAMP to elongate its half-life. For this reason blockade of cAMP synthesis by betaxolol diminish the activity of silibinin. Cyclic AMP was found to inhibit transepithelial  $Cl^-$  secretion across bovine ciliary epithelium by uncoupling the intracellular gap junction<sup>(23)</sup> and to inhibit other important regulator of

aqueous humor dynamics, the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter<sup>(24)</sup>. Inhibition of this cotransporter has found to be associated with higher degree of reduction in IOP. Interestingly, this profile of activity is similar to that produced by 0.75% silybinin (38.5%) in the present study. Although the effect of silibinin on the ocular phosphodiesterase (PDE) has not been studied, a study on beef heart PDE revealed that silibinin was more potent as PDE-inhibitor than theophylline and papaverine in this regard<sup>(13)</sup>. From these findings one can suggest that the strong ocular hypotensive effect produced by silibinin might be attributed to the inhibition of PDE, and the resultant accumulation of cAMP inhibits Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter 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 silibinin produces this effect, and became a new drug candidate for the reduction of elevated IOP. In conclusion, the results obtained in this study provide experimental evidence that silibinin is more potent in reducing IOP than betaxolol through a mechanism that might be related to increase cAMP levels.

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