

Research Article



Tolerable Ocular Hypotensive Effect of Topically Applied Sildenafil in Ocular in Normotensive and Betamethasone-Induced Hypertensive Rabbits

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Accepted on: 19-09-2015; Finalized on: 31-10-2015.

ABSTRACT

The study aimed to evaluate the effect of topical sildenafil drop on Intraocular Pressure (IOP) in rabbits of both normotensive and betamethasone-induced hypertensive eyes. 36 rabbits allocated into two groups: ocular normotensive and hypertensive group, each group further subdivided into 3 subgroups. Sildenafil ophthalmic solution (0.3%) was instilled twice daily for 7 days in normotensive and hypertensive subgroups, another two subgroups of both normotensive and hypertensive were used to evaluate the effect of timolol and distilled water (DW). Intraocular pressure IOP measured by Schiottz tonometer. Sildenafil was very effective in lowering IOP in both normotensive & ocular hypertensive rabbits. Mean IOP decreased after one day of sildenafil instillation (2.5 ± 0.56 mmHg) in normotensive eye and (1.3 ± 0.61) in hypertensive eyes. A significant results ($P \leq 0.05$) were obtained when compare the results of sildenafil with DW; no significant differences were obtained with timolol group. Sildenafil ophthalmic solutions have the capability of IOP reduction and possible modulation of its regulatory mechanisms.

Keywords: IOP, glaucoma, sildenafil, timolol, ocular hypertension.

INTRODUCTION

Glaucoma is the second leading cause of vision loss in the world and its predominance increases with certain risk factors, including older age and elevated intraocular pressure (IOP)¹. Slowing or stopping the progression of the disease by lowering the IOP is the only proven method². Chronic open-angle glaucoma is the most prevalent form of this disease in western countries, which is estimated to have affected about 33 million people worldwide by the year 2000¹. New antiglaucoma agents seem to be needed in view of the magnitude of the glaucoma problem and the paucity of drugs available to treat it. Frequently used classes of ocular hypotensive medications include beta-adrenergic antagonists (beta-blockers), prostaglandin analogs (including prostamides), alpha-adrenergic agonists, and carbonic anhydrase inhibitors³. Phosphodiesterase 5 (PDE5) inhibitors are the most effective oral treatment agent for patients with erectile dysfunction. These agents inhibit the PDE5 enzyme, which breaks down cyclic guanosine monophosphate (cGMP) in all vascular tissue, the secondary messenger of nitric oxide. The inhibition of PDE5 potentiates the muscle-relaxant effect of nitric oxide and cGMP. Moreover, PDE5 inhibitors have a weaker inhibitory action on phosphodiesterase 6 (PDE6)⁴. Sildenafil is the first and most well-known PDE5 inhibitor.⁵ The aim of this study was to evaluate the effects of topical application of sildenafil on intraocular pressure (IOP) of ocular normotensive and betamethasone-induced hypertensive rabbits and thus to determine the effects of the drug on the eye for the first time in the literature. Corticosteroid glaucoma is among experimental models more closely resembling human

disease, since both its clinical features (elevated IOP and gonioscopically open-angle) and underlying mechanism (reduced aqueous outflow) mimic those of human chronic open-angle glaucoma. In contrast to most of the induced experimental models for glaucoma, corticosteroid glaucoma is also observed in ophthalmological practice after topical, periocular or systemic administration of corticosteroids, a fact that strengthens the parallel between the animal and human disease. Furthermore, evidence suggesting that endogenous glucocorticoids may play a role in the development of ocular hypertension in humans seems to support the utility of this glaucoma model⁶.

METHODS

Animals

Thirty six male albino rabbits weighing 1.5-2.5 kg with no signs of ocular inflammation or gross abnormality were used in this study. The animals allocated into 2 groups represent the ocular normotensive and induced-hypertensive groups. Each group further subdivided into 3 subgroups with 6 animals in each subgroup. Animals in one subgroup receive sildenafil while the other 2 subgroups receive timolol and distilled water (DW), respectively. Animals were housed individually in plastic cages; all rabbits were maintained conventionally during the study with regulated air temperature (15-21 °C), artificial light cycle (12 hours light/12 hours darkness) and good ventilation. They fed standard rabbit diet and had free access to drinking water. Animal experiments conformed to the ARVO Statement for the Use of Animals in Research and approved by the Alnahrain University Graduate and Ethical Committee.



Preparation of sildenafil ophthalmic solution

Ophthalmic solutions are isotonic, sterile, free from foreign particles, and specially prepared for instillation in the eye⁷. Ophthalmic solution was prepared by dissolving the desired amount of sildenafil powder in appropriate volume of phosphate buffer solution with subsequent addition of sufficient amount of NaCl to make solution isotonic. These mixtures were then stirred well using electronic ultrasonic shaker to accelerate the dissolution of the undissolved particles, the benzalkonium chloride solution was added and mixed well prior to addition of phosphate buffer solution to achieve the final volume. Adjustment of solution pH to (7.4) either by addition of few drops of phosphoric acid or sodium hydroxide solution. The prepared ophthalmic solution then filtered through a disposable micro-filter paper to be ready for instant instillation. The ophthalmic solution prepared in a septic condition then filled in sterile containers⁸. The formula was prepared as shown in the following: Sildenafil 0.5g, Benzalkonium chloride (1% w/w) 1ml, Sodium citrate 0.45g, Ethanol 1ml, Phosphate buffer to 100 ml. This formula for the preparation of sildenafil according to British pharmacopeia 2002.

IOP measurement

After local anesthetization of the cornea with 1-2 drops of 4% lidocaine HCL drop, the animal was hold on his back and Schiotz tonometer is placed on the cornea. A control or zero time value of IOP was taken 15 minutes before the administration of sildenafil. One drop of freshly prepared sildenafil was instilled in the middle of inferior conjunctival sac followed by lid closure. Thereafter, IOP was measured after 1 hour of topical application of tested drug. Tested drug instilled as one drop (50µl) twice daily for 7 days and IOP measured daily at about the same time (9:00 am) to avoid diurnal IOP fluctuation. After each measurement the instrument was properly cleaned with diethyl ether. Left eye of the rabbits were used for evaluation of the tested drugs. On the other hand, right eye were considered as a control in which DW was instilled for comparison with left eye if there is contralateral effect of sildenafil or any side effect, change in pupil diameter and conjunctival redness of topical application of sildenafil. To suppress growth of bacteria and other microorganism being introduced by the device or during drug administration, ophthalmic chloramphenicol eye drop preparation was instilled in the eye at the end of each experiment.

Pupil diameter

The measurement of pupil diameter was done for both eyes of rabbit by using the pupil gauge. The obtained results would be presented in millimeter unit⁹.

Light reflex

The light reflex or pupillary response of both eyes was tested by swinging flashlight to detect a relative afferent pupillary defect. The obtained results would be presented

as either it was intact or absent¹⁰.

Corneal reflex

It could be tested for both eyes by using wisp of cotton wool, it applied from the side and award of its approach. The obtained results would be presented as either it was intact or absent⁹.

Conjunctival redness

It could be detected by inspection of conjunctiva of both eyes. The obtained results would be presented as either it was present or not¹¹.

Lacrimation

It could be detected by inspection of conjunctiva of both eyes. The obtained results would be presented as either it was present or not¹¹.

Application of Sildenafil, Timolol and DW on Ocular Normotensive Rabbits

Animals were divided into groups each group included six rabbits. Tested drug instilled as one drop (50µl) twice daily for 7 days in normotensive left eye and the groups of this part of the study include:

- i. Isotonic Phosphate Buffer group.
- ii. Timolol maleate (0.3 %) group.
- iii. Sildenafil (0.5%) group.

Application of the Sildenafil, Timolol and DW on the induced -Ocular Hypertensive Rabbits

Ocular hypertension was done in the left eye, while the right eye left without induction. After proper anesthetization of the left eye by local instillation of 4% lidocaineHCl, subconjunctival injection (by using micro-fine syringes 30 gauge x1/2 inches) of 0.7 ml of betamethasone suspension (CelestoneChronodose; Schering-Plough, Spain) containing betamethasone sodium phosphate (3 mg/ml) and betamethasone acetate (3 mg/ml). This formulation provides a readily accessible (sodium phosphate) and a sustained release (acetate) fraction of betamethasone. To avoid corneal epithelium damage through too-frequent tonometry, measures of IOP in both eyes were as a rule repeated twice a week, with the first measure being taken immediately before the weekly betamethasone subconjunctival injection and the second taken after 3 days. Three base-line IOP measures were recorded during the week before betamethasone treatment, with animals exhibiting fluctuations of > 2 mmHg excluded from the experiments. The value observed at zero time (first betamethasone injection) was considered the starting pressure. All the animals received weekly subconjunctival injections of betamethasone into the left eye over a period of 4 weeks. The instillation of the tested drugs were started at the 24th day of corticosteroid treatment (3 days after the fourth subconjunctival injection), a time at which the betamethasone-induced ocular hypertension turned out



to be stable, and was prolonged up to 25 days. The experiment model that produced is mimic human chronic open angle glaucoma¹².

Tested drug was instilled as one drop (50µl) twice daily for 7 days (only after the ocular hypertension was definitely established). Each group of this study included six rabbits and the groups of this part included:

- i. DW group.
- ii. Timolol maleate (0.5 %) group.
- iii. Sildenafil (0.5%) group.

Statistical analysis

The obtained data were presented as mean±SEM (standard error of mean). The results were analyzed statistically using Student paired *t*-test for assessing the effect of employed drug for a given group of patients. While Student (unpaired) *t*-test for independent data was used to test the significance of the difference between any two groups. Differences were accepted as insignificant if $P>0.05$ and significant if $P\leq 0.05$ ¹³.

RESULTS

The effect of timolol on pupil diameter, light reflex, corneal reflex, conjunctival redness & lacrimation of hypertensive rabbits eye and the effect of Sildenafil on pupil diameter, light reflex, corneal reflex, conjunctival redness & lacrimation of normotensive rabbits eye were shown by tables 1 and 2 respectively.

Table 1: Effect of timolol on pupil diameter, light reflex, corneal reflex, conjunctival redness & lacrimation of hypertensive rabbits eye.

Parameter	Effect
Pupil diameter	No effect
Light reflex	No effect
Corneal reflex	No effect
Conjunctival redness	No Conjunctiva redness
Lacrimation	No Lacrimation

Table 2: Effect of Sildenafil on pupil diameter, light reflex, corneal reflex, conjunctival redness & lacrimation of normotensive rabbits eye.

Parameter	Effect
Pupil diameter	No effect
Light reflex	No effect
Corneal reflex	No effect
Conjunctival redness	No Conjunctival redness
Lacrimation	No Lacrimation

Part I

Effect of Tested Drugs on Normotensive eyes

Effect of Isotonic Phosphate Buffer

Response of mean IOP (figure 1)

The effect of isotonic phosphate buffer (vehicle) used for preparation of ophthalmic solution of the tested drugs on mean IOP of rabbits eyes did not reach the level of statistical significant ($P\leq 0.05$) during the time course of the experiment (7 days).

Effect of Distilled Water (Negative Control Group)

Response of mean IOP (figure 1)

Effect of DW on mean IOP of rabbits eyes nearly remained constant during the time course of experiment ($P>0.05$).

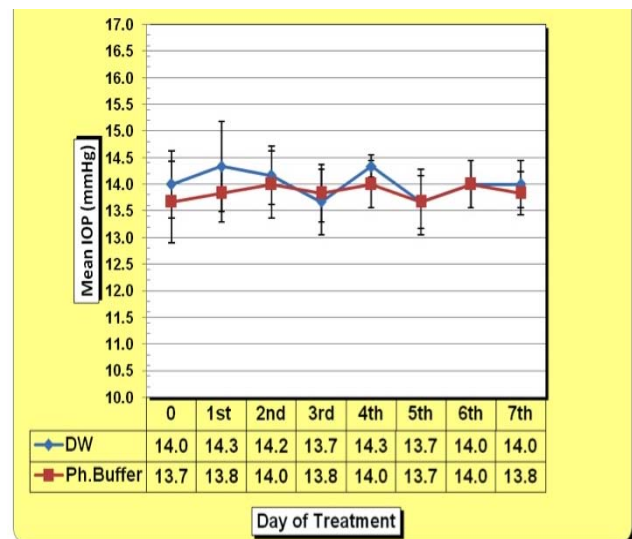


Figure 1: Effect of isotonic phosphate buffer and DW on mean IOP (mmHg) of left and right eyes respectively in rabbits (n=6/group).

Effect of Timolol (0.5%) Drop

Response of mean IOP (Figure 2)

The mean IOP value prior commencement of timolol instillation (0 time or pretreatment value) was ((14.2±0.42 mmHg). After 1 hour of single drop instillation of the timolol, the mean IOP value decreased to be (12±0.57mmHg), such reduction was found to be significant ($P=0.02$). Compared to that of DW significant mean IOP reduction ($P=0.03$) was found. After 1 day of timolol instillation (2 times/day), mean IOP significantly decreased by (2.5±0.67mmHg; $P=0.013$). Maximum mean IOP reduction was (3.2±0.75 mmHg) that achieved on day 7, such reduction was found to be significant ($P=0.008$). Compared to that of DW a significant mean IOP reduction ($P\leq 0.05$) was found on day 1, 2, 3, 4, 5, 6 and 7.

The mean IOP value prior commencement of sildenafil instillation (0 time) was (15.2 ± 0.48 mmHg). After 1 hour of single drop instillation, the mean IOP value decreased to be (13.7 ± 0.61 mmHg), such reduction was found to be insignificant ($P = 0.08$) when compared to pretreatment value. Compared to that of DW there was no significant difference ($P=0.55$). Compared to that of timolol there was no significant effect ($P = 0.09$) in mean IOP reduction. After 1 day of sildenafil instillation (2 times/day), mean IOP significantly ($P = 0.007$) decreased by (2.5 ± 0.56



mmHg). Maximum mean IOP reduction was (4.3 ± 0.49 mmHg) that achieved on day 6, such reduction was found to be significant ($P = 0.0003$). Along all trial period there are significant ($P \leq 0.05$) mean IOP reductions when compared to pretreatment value. Compared to that of DW a significant mean IOP reduction ($P \leq 0.05$) on day 2, 3, 4, 5, 6 and 7. Compared to that of timolol there was no significant effect ($P > 0.05$) along all trial period. Compared to that of sildenafil there was no significant effect ($P > 0.05$) along all trial period.

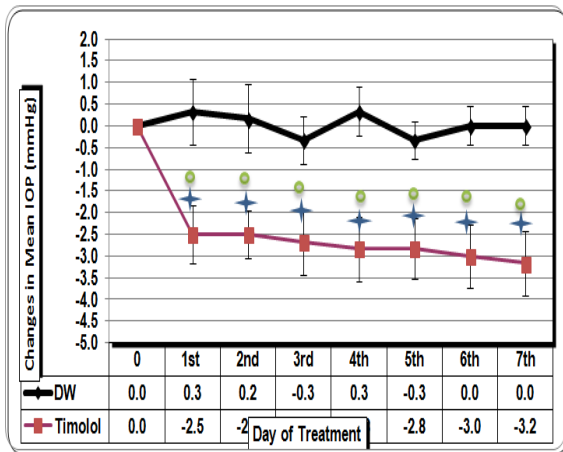


Figure 2: Degrees of timolol (0.5%)-induced changes in mean IOP (mmHg) of ocular normotensive rabbits eyes (n=6/group). \star denotes significant difference when compared with pretreatment value; \circ denotes significant difference when compared to DW.

Effect of Sildenafil (0.3%) Ophthalmic Solution

Response of mean IOP (Figure 3)

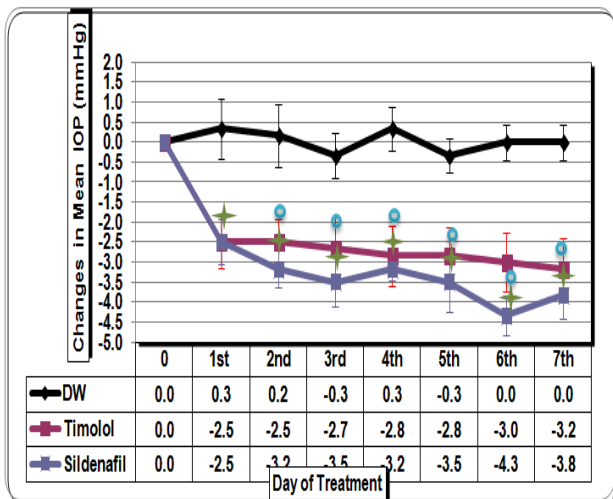


Figure 3: Degrees of sildenafil (0.5%)-induced changes in mean IOP (mmHg) of ocular normotensive rabbits eyes (n=6/group). \star denotes significant difference when compared with pretreatment value; \circ denotes significant difference when compared with timolol.

Part II

Effect of tested drugs on experimentally induced ocular hypertension

The mean IOP of rabbits receiving weekly sub-conjunctival injections of betamethasone suspension for four weeks, gradually increased throughout the experimental period, which became statistically significant since the third day ($P=0.0001$) and reached its maximum at the end of the fourth week. This model of induction produced ocular hypertension mimic human chronic open- angle glaucoma.

Effect of Distilled Water (Negative Control Group)

Response of mean IOP (Figure 4)

Prior to induction of ocular hypertension the mean IOP was (15.5 ± 0.47 mmHg). At post induction of ocular hypertension (i.e. after 4 weeks of sub-conjunctival betamethasone suspension injection) prior to instillation of distilled water, the mean IOP was (21.5 ± 0.33 mmHg); then after DW instillation (twice daily), the mean IOP did not reach the level of statistical significant ($P \geq 0.5$) during the time course of the experiment. On the other hand, there are no effect of DW on pupil diameter, light reflex, corneal reflex and no conjunctival redness, lacrimation or purulent discharge.

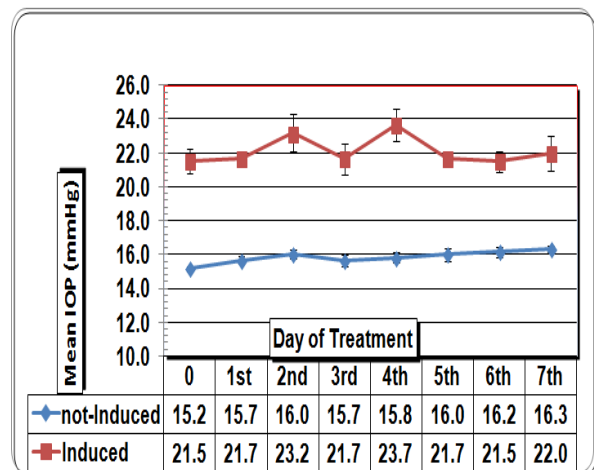


Figure 1-4: Effect of DW on mean IOP (mmHg) of induced-ocular hypertensive eyes in rabbits (n=6/group)

Effect of Timolol (0.5%) Drop

Response of mean IOP (Figure 5)

At post induction of ocular hypertension, the mean IOP was (24 ± 0.98 mmHg). After one hour of single drop instillation of timolol (0.5%), mean IOP reduced to be (21.7 ± 1.18 mmHg), such reduction was found to be not significant ($P = 0.06$) when compared to pretreatment value. Compared to that of DW there is no significant effect of timolol ($P=0.2$). After 1 day of timolol instillation (twice daily), the reduction in mean IOP was (5.0 ± 0.77 mmHg) which was found to be significant ($P=0.004$). Decline in mean IOP was found to be significant in days 2 ($P=0.02$), 4 ($P=0.03$), 5 ($P=0.02$), 6 ($P=0.01$) and day 7 ($P=0.01$). Compared to that of DW a significant mean IOP reduction ($P \leq 0.05$) was observed on day1, 2, 4, 5, 6 and 7.

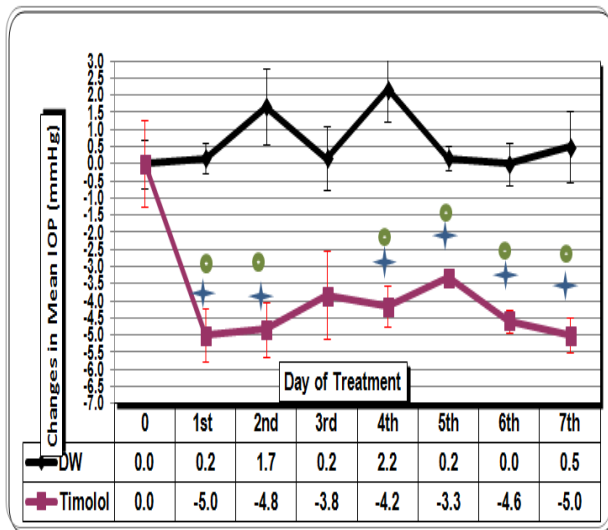


Figure 5: Degrees of timolol (0.5%) - induced changes in mean IOP (mmHg) of ocular hypertensive rabbits eyes (n=6/group). \star denotes significant difference when compared with pretreatment value; \circ denotes significant difference when compared to DW.

Effect of Sildenafil (0.3%) Ophthalmic Solution

Response of mean IOP (Figure 6)

At post induction of ocular hypertension, mean IOP was (21.5 ± 0.96 mmHg). After one hour of single drop instillation of sildenafil, mean IOP significantly (P = 0.02) reduced to be (19.4 ± 0.66 mmHg). Compared to that of DW, sildenafil more effective (i.e. significant) (P = 0.009). Compared to that of timolol there was no significant difference (P = 0.2). After one day of treatment with sildenafil (twice daily), mean IOP decreased by (1.3 ± 0.61 mmHg), such reduction was found to be insignificant (P = 0.06). On day 2 a significant (P = 0.03) mean IOP reduction was found (2.7 ± 0.95 mmHg), in day 3 maximum mean IOP decline was achieved (3.3 ± 1.41 mmHg).

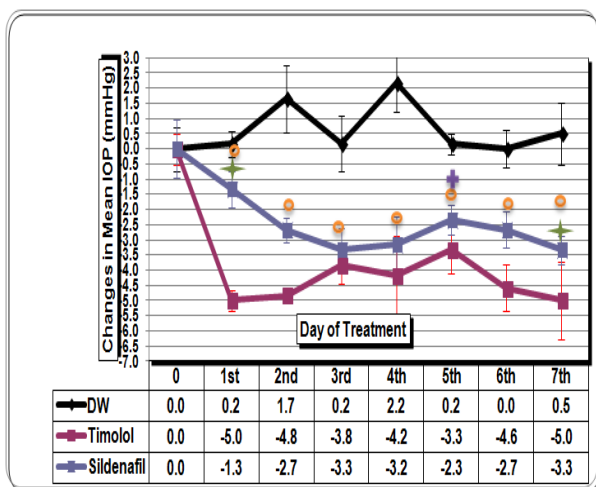


Figure 6: Degrees of sildenafil (0.5%) - induced changes in mean IOP (mmHg) of ocular hypertensive rabbits eyes (n=6/group). \star denotes significant difference when

compared with pretreatment value; \circ denotes significant difference when compared to DW; \star =Significant difference when compared with timolol.

Compared to that of DW group, sildenafil more efficient in days 2 (P = 0.004), 3 (P = 0.01), 4 (P = 0.002), 5 (P = 0.001), 6 (P = 0.008) and day 7 (P = 0.02). Compared to that of timolol group, no significant difference (P > 0.05) detected in mean IOP along the trial period except in day 5 where was timolol more efficient than sildenafil (P = 0.01).

DISCUSSION

Effect of DW (Negative Control Group)

DW considered as an inert agent that used widely as a negative control for testing the activity of the induction of ocular hypertension and the efficacy of the tested drugs^{14,2,15}.

In the present study, twice daily instillation of DW for 7 days produced no significant change in mean IOP in both normotensive and induced ocular hypertensive (P>0.05), these results are in agreement with Heijl² and Kitazaw¹⁵.

Effect of Isotonic Phosphate Buffer (Vehicle)

Isotonic phosphate buffer that used in preparing ophthalmic solution of the tested agents also produced no significant change in mean IOP in both normotensive and induced ocular hypertensive when instilled twice daily for 7 days these results strengthened by Allen⁸ and Kramer¹⁶ who found the effect of inactive ingredients as a supporting materials have no change in the IOP reading.

Effect of Timolol (0.5%) eye Drop

Timolol eye drop was used as positive control to test the ocular hypotensive effect of most experimented drugs and its preferred in chronic open glaucoma¹⁷. The present study obviously indicated that timolol produced a considerable reduction in the mean IOP. The obtained reduction in mean IOP after 1 hour was (15.3%) in ocular normotensive and (9.7%) in hypertensive models. On other hand, decline in mean IOP that obtained after 1 day was (17.65%) in normotensive and (20.83%) in hypertensive models. Maximal mean IOP reduction was achieved after 7 days of installation in normotensive eyes (22.35%), while, maximal IOP reduction (20.83 %) achieved after 1 day in ocular hypertensive. These results are strengthened by Mikael¹⁸, Gupta and co-workers¹⁹ reported that timolol (0.5%) produced (20%) to (35%) reduction in mean IOP in ocular hypertensive eyes. Timolol drop application in the present study had no effect on pupil diameter (table1) and no effect regarding other possible side effect (i.e. light reflex, corneal reflex and conjunctival redness), these results agreed by Mikael¹⁸. Most of β - adrenergic antagonists decrease the production of AH by about one third²⁰. Timolol act solely by reducing the AH production by its action on ciliary body²¹ while leaving AH outflow unchanged²². Charles and co-workers²³ had suggested an alternative

mechanism by which timolol can act independently of cAMP-mediated pathways, timolol could inhibit AH secretion by blocking Cl⁻ uptake by the PE cells at the stromal surface, thereby reducing intraepithelial Cl⁻ content. This supports the idea that timolol acts at the stromal side to block NaCl uptake by the paired antiports, Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers²⁴. Further support was provided by the observation that reducing delivery of HCO₃⁻ and H⁺ to the paired antiports (by inhibiting carbonic anhydrase) and directly inhibiting the Na⁺/H⁺ antiport (with dimethyl amiloride) mimicked the change in intracellular composition caused by timolol²³.

Effects of Sildenafil (0.3%) Ophthalmic Solution

Al-Shureifi²⁵ approved that single instillation of (0.25 %) and (0.35 %) sildenafil ophthalmic solution were able to reduce IOP significantly in normotensive rabbit's eyes. In this study (0.3 %) concentration was selected to evaluate its IOP-lowering effect on induced ocular hypertensive and normotensive rabbits eyes. The present study clearly demonstrated that single drop of sildenafil able to reduce mean IOP by (9.89%) in ocular normotensive and by (9.69%) in hypertensive models after one hour of instillation. On the other hand, sildenafil was able to reduce mean IOP by (16.48%) in normotensive, and (6.25%) in hypertensive rabbits eyes after 1 day of instillation. Maximal mean IOP reduction (28.57%) achieved in day 6 of normotensive and (15.5%) achieved in day 4 of hypertensive eyes. The hypotensive effect of sildenafil was valid when compared with DW and comparable to that of timolol except a more pronounced hypotensive effect of timolol in day 5. Along the trial period sildenafil had no significant effect on IOP of untreated eyes and there was no contralateral effect. Sildenafil drop application in the present study had no effect on pupil diameter (table 2) and no effect regarding other possible side effect (i.e. light reflex, corneal reflex and conjunctival redness), these results agreed by Mikael¹⁸.

Sildenafil is a highly selective and competitive inhibitor of PDE5²⁶, the chemical structure of sildenafil is very similar to cGMP and therefore, it competitively binds to the catalytic site of PDE5 enzyme, preventing the PDE5 breakdown of cGMP²⁷.

Cyclic GMP is a key signal transduction messenger in the regulation of several physiologic processes, including smooth muscle relaxation, through its ability to stimulate cGMP-dependent protein kinase (PKG) which in turn phosphorylate cellular protein substrate and evoke specific responses such as reduction in intracellular calcium concentration and inhibition of Na⁺/K⁺-ATPase in NPE thereby IOP will be reduced²⁸. Both cAMP and cGMP have been implicated in control of IOP²⁹. The PDEs vary in their substrate specificity for them³⁰. Zhou and co-workers³¹ identified PDE4, 5, and 7 in human trabecular meshwork cells and this strongly asserted the possibility that selective PDE inhibitors might be used to control elevated IOP in glaucoma. Findings in this study come in

accordance with previous studies in which increasing the production of cGMP by cGMP analogues (8-bromo-cGMP), NO donors (sodium nitroprusside) or Guanylyl cyclase activators (atriopeptin) lower IOP in animals (decrease AH inflow)³²⁻³⁴. Furthermore, Al-Shureifi²⁵ approved that sildenafil instillation will decrease IOP significantly in normotensive eyes of rabbits. Thus, sildenafil could be considered as an inhibitory modulator of AH production by decreasing intracellular Ca²⁺³³. In addition to above, sildenafil could increase AH outflow and uveoscleral outflow^{35,36}.

Other suggested mechanism by which sildenafil could inhibit AH secretion by blocking Na⁺/K⁺-ATPase in NPE as a result of cGMP elevation³⁷. This mechanism fits with Ellis³⁸ and Ellis and co-workers³⁹ that reported elevation of cGMP inhibit Na⁺/K⁺-ATPase in choroid plexus and CP, thus, IOP will decrease.

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Source of Support: Nil, Conflict of Interest: None.

