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Topical effects of atorvastatin on induced ocular hypertension in rabbits

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Abstract

Glaucoma defined as a group of optic neuropathies characterized by elevated intraocular pressure which is the most important factor and progressive retinal ganglion cells degeneration. Atorvastatin has an intraocular pressure lowering effect by blocking the Rho Kinase signaling pathway by increase aqueous humor outflow by reversing structural and functional damage at the trabecular meshwork leading to vasodilation and decrease the episacral venous pressure. The present study was conducted to evaluate the intraocular lowering effect, anti-oxidant and anti-inflammatory effects of atorvastatin in ocular hypertensive in rabbits.Group of (40)adult male of New Zealand rabbits, were divided to 3 groups: isotonic buffer group (8 normotensive rabbits), this group was instilled with isotonic buffer solution in the right eye and distilled water in the left eye to show if there is any effect of the vehicle (isotonic solution) on the eye, latanoprost group(8 rabbits) were both eyes of this group have been induced for ocular hypertensive, the right eyes instilled with latanoprost (0.005%) drop once daily which considered as a positive control group, while the left eyes instilled with DW once daily which considered as a negative control group., atorvastatin group (24 rabbits), divided in to (3) subgroups 8 rabbits in each (0. 5%, 1%&2%) that instilled once daily. The results obtained in this study provide experimental evidences for the effectiveness of atorvastatin (0.5%, 1%, 2%) ophthalmic solutions on the reduction of mean IOP and have an antioxidant and anti-inflammatory effect in induced ocular hypertensive eyes in rabbits.

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Introduction

Glaucoma defined as a group of optic neuropathies characterized by progressive retinal ganglion cells (RGCs) degeneration (Weinreb *et al*., 2014) resulted from fluid builds up in the front part of the eye. That extra fluid increases the pressure in the eye (Kierstan, 2017). According to The World health Organization, glaucoma accounted for 2 percent of visual impairment and 8 percent of global blindness in 2010(WHO, 2017). It is a family of related diseases frequently associated with elevated IOP which is exceeds 21 mmHg and may be as high as 70 or 80 mmHg during the attack (Jay and Murdoch, 1993). Many factors lead to retinal ganglion cell death (RGC death) include; elevated intraocular pressure, vascular dysregulation and oxidative stress (Kaushik *et al*, 2003).

The production and drainage of aqueous humor must be in Equilibrium to maintain the intraocular pressure within the normal range despite that disruption in aqueous inflow, outflow, or both, results in raise of intraocular pressure , which is a major risk factor in the pathogenesis of glaucoma (Goel *et al*., 2010).Open-angle glaucoma which is the most common form of glaucoma, accounting for at least 90% of all glaucoma cases (glaucoma research foundation ,2017). Reduction of intraocular pressure is the primary goal in patients with glaucoma (Chang and Goldberg, 2012). However, even with treatment to lower IOP and even in normal tension glaucoma optic nerve damage may progress (McKinnon *et al*., 2008). There is an excessive formation of free radicals and oxidative stress is recognized as an etiopathogenetic factor and a significant depletion in antioxidant potential in glaucoma-affected patients (Ferreira *et al*., 2004).

Neuroprotection in glaucoma is aimed at protecting those neurons that are damaged or likely to be damaged in glaucomatous optic neuropathy which consists of neurons along the entire visual pathway, chiefly the RGC axons. This strategy is an addition to that achieved by IOP lowering alone. Even though any treatment approach that preserves RGCs in glaucoma could be described as neuroprotective (Weinreb and Levin, 1999).

The intraocular pressure lowering effect of Atorvastatin may be by blocking the Rho/ROCK signaling pathway due to the human trabecular meshwork expresses many components of the Rho pathway, suggesting that this is a key player in regulating the contractile tone and cellular morphology of the aqueous outflow pathway (Rao *et al*., 2001). Another possible lowering effect of atorvastatin is in the eye depend on its effect on the endothelium-derived nitric oxide (NO) which is an important regulator of blood flow to the choroid, optic nerve and retina (Luksch *et al*., 2000). The raised levels of GSH in atorvastatin treatment is a dose response effect due to the antioxidant mediated effect of atorvastatin which result from inhibition of mevalonate pathway leading to the reduction in the synthesis of important intermediates including isoprenoids (farnesyl pyrophosphate & geranylgeranyl pyrophosphate) which serve as lipid attachments for intracellular signaling molecules in particular inhibition of small GTPase binding proteins (Rho, Rac, Ras and G proteins). These proteins modulate a variety of cellular processes including signaling, differentiation and proliferation (Mason, 2003)(Liao and Laufs, 2005). The anti-inflammatory effect of atorvastatin result in decreased TNF-induced Rac-1 membrane translocation and activation, intracellular ROS production and expressions of its downstream inflammatory cytokines such as neuclear factor-kB (NF-kB) , vascular cell adhesion molecule -1(VCAM-1) and intracellular adhesion molecule -1 (ICAM-1) (Ohnesorge *et al*., 2010).The present study aimed to evaluate the possible IOP-lowering effect of corneal instillation of atorvastatin in experimentally corticosteroid induced- ocular hypertensive eyes of rabbits and To compare the dose-response curve of atorvastatin with latanoprost in lowering IOP. In addition to explore the antioxidant and anti-inflammatory activity of atorvastatin in the aqueous humor in rabbits with induced ocular hypertension and to explore the possible side effects of atorvastatin

on eyes after instillation.

Materials and methods

Group of (40) adult male of Zealand rabbits weighing 1.5 to 2 kg with no signs of ocular inflammation or gross abnormality were used in this study. Isotonic buffer group (8 normotensive rabbits) was instilled with isotonic buffer solution in the right eye and DW in the left eye to show if there is any effect of the vehicle (isotonic solution) on the eye. Latanoprost group(8 rabbits) both eyes of this group have been induced for ocular hypertensive, the right eyes instilled with latanoprost0.005% drop (1-2drop) once daily which considered as a positive control group, while the left eyes instilled with DW which considered as a negative control group. Atorvastatin group (24 rabbits), divided into (3) subgroups (8 rabbits): instilled with Atorvastatin 0.5% drop (8 rabbits): instilled with Atorvastatin 1% drop (8 rabbits): instilled with Atorvastatin 2% drop.

The right eyes of these groups were induced for ocular hypertensive and instilled with (1drop) of Atorvastatin drop once daily for 7 days. These concentrations were chosen after doing a pilot study on 6 animals using different concentrations of the tested drugs and the used concentrations were chosen depend on the effect and adverse effect. Animals were housed individually in aplastic cages; all rabbits were maintained conventionally during the study with regulated air temperature (15-21 c), an artificial light cycle (12 hours light /12 hours darkness) and good ventilation. They fed a standard rabbit diet and had free access to drinking water (Mohammad *et al*, 2018).

Induction of ocular hypertension

According to Melana *et al*., (1998) who found that this model of induction of Ocular hypertension is mimic human chronic open angle glaucoma. the procedure as following: After proper anesthetization of eyes by local instillation of 2% lidocaine HCL , a subconjunctival injection (by using a micro-fine syringes, 30 gauge × 1/2 inches) of 0.7 ml of betamethasone suspension containing betamethasone sodium phosphate (3 mg/ml) and betamethasone acetate (3 mg/ml).using this formulation provides a readily accessible (sodium phosphate) and a sustained release (acetate) fraction of betamethasone. Measures of IOP repeated twice a week to avoid corneal epithelium damage through too-frequent tonometry. The first measure being taken immediately before the weekly betamethasone subconjunctival injection and the second was taken after 3 days. Three base-line IOP measures were recorded during the week before betamethasone treatment. The value observed at zero time (first betamethasone injection) was considered the starting pressure. The animals received weekly subconjunctival injections of betamethasone over a period of 21 days (four doses).

The instillation of the tested drugs was started at the 24th day of corticosteroid treatment (3 days after the fourth subconjunctival injection). Tested drug was instilled in the right eyes as one drop (50µl) for 7 days twice for metformin (only after the ocular hypertension was definitely established).

Preparation of Atorvastatin ophthalmic solution

In the preparation of aqueous ophthalmic drops, a careful consideration of the need for isotonicity, a certain buffering capacity, the desired pH, the addition of antimicrobial agents and/or antioxidants, the use of viscosity-increasing agents, and the choice of appropriate packaging (The International Pharmacopoeia,2016). Ophthalmic solutions are isotonic, sterile, free from foreign particles, and specially prepared for instillation in the eye (Hecht, 2000).

Intraocular pressure measurement

After local anesthetization of the cornea with 1-2 drops of 2% lidocaine HCL ophthalmic solution, the animal was hold and Schiotz tonometer is placed on the cornea.

A control or zero time value of IOP was taken 15 minutes (min) before the administration of tested drug. One drop of freshly prepared tested drug 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.

Metformin instilled as one drop (50µl) for 7 days twice daily and IOP measured daily at about the same time to avoid diurnal IOP fluctuation. cleansing the instrument with diethyl ether after each measurement properly to suppress growth of bacteria and other microorganism being introduced by the device or during drug administration, ophthalmic eye drop preparation containing a suitable antibacterial agent (chloramphenicol eye drop about 1-2 drops) were instilled in the eye rabbits at the end of each experiment (Moses,1997).

Pupil diameter

Pupil diameter measurement was done by using the pupil gauge. The obtained results represented in millimeter unit (Ahuja, 2003).

Light reflex

Pupillary response or the light reflex of both eyes was tested by swinging flashlight to detect a relative afferent papillary defect. The obtained results would be presented as either it was intact or absent (Jampel, 2001).

Corneal reflex

Both eyes were tested 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 absent (Ahuja , 2003).

Conjunctival redness

Both eyes were tested to detect by inspection of conjunctival redness. The obtained results would be presented as either it was present or not (Macdonald, 2000).

Lacrimation

It could be detected by inspection of conjunctival lacrimation of both eyes. The obtained results would be presented as either it was present or not (Macdonald, 2000).

Aqueous humor collection

This step was done for the measurement of the antioxidant and the anti-inflammatory effects of the tested drugs. Aqueous humor was collected carefully from the anterior chamber of rabbit's eye using 27-gauge needle after administration of diazepam 1 mg/kg i.v. and ketamine 25 mg/kg i.v. without causing any injury to the iris or lens throughout the procedure (Gupta *et al*., 2011). the onset of anesthesia provided by ketamine begin after six minutes and the peak effect after ten minutes which persist about forty minutes (Green *et al*,1981), the combination of ketamine with diazepam produce surgical anesthesia in rabbit that continue for up to 30 minutes (Flecknell, 2009). After collection, samples were immediately stored at temperature (–20°C) till the performance of various biochemical analysis from AH (Gupta *et al*., 2011).

Glutathione (GSH) determination in aqueous humor samples

This test was performed for the determination of the glutathione (GSH) levels in rabbits aqueous humor.The ELISA kit used is a competitive-ELISA.

Tumor necrosis factor alpha determination (TNF-α) in aqueous humor samples

This test was performed in mini market research and consumer center laboratories for the determination of the tumor necrosis factor alpha (TNF-α) levels in rabbits aqueous humor. The kit used is based on sandwich enzyme-linked immune-sorbent assay technology

Statistical analysis

The results were presented as means ± standard error (SEM). One way analysis of variance (ANNOVA) followed by Tukey test comparison t-test (2-tailed) was utilized to compare between groups. The differences between the means are studies as significant at the 0.05 confidence level. This done by Microsoft excel 2016 and SPSS version 23. The concentration that decreases 50% of the IOP this value was analyzed by linear regression equation and logarithmic equation. The level of significance was set

at P<0.05 as significant (Daniel and Yu, 2008).

Results

Effect of Isotonic buffer solution and DW on normotensive rabbits eyes (Response of mean intraocular pressure (IOP))

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

Table 1. Glutathione level in control positive with other study groups by unpaired t-test.

Glutathione (Mg/ml)	Control+ve (latanoprost 0.005%)	Control-ve(healthy untreated group)	Atorvastatin 0.5%	Atorvastatin 1%	Atorvastatin 2%
Mean±SD	5.3±2.05	5.7±2.31	9.1±7.18	13.91±7.6	14.42±2.11
P value		0.755*	0.241*	0.038*	<0.001*
				0.008**	

*p value of comparison between control +ve and other study groups by unpaired t- test,
**p value of comparison among six groups (apart from control) by ANOVA.

Effect of Distilled Water

Response of mean IOP: Effect of DW on mean IOP of rabbits left eyes nearly remained constant during the time course of experiment (P = 0.949) (Fig 1). Isotonic buffer solution and DW application in the present study had no effect on pupil diameter and no effect regarding other possible side effect (i.e. light reflex, corneal reflex, conjunctival redness and lacrimation).

Table 2. Comparison of Tumor necrosis factor alpha levels in control positive with other atorvastatin groups by unpaired t-test.

TNFα (pg/ml)	Control+ve (latanoprost 0.005%)	Control-ve (healthy)	Atorvastatin 0.5%	Atorvastatin 1%	Atorvastatin 2%
Mean±SD	15.1±3.46	13.3±0.49	10.45±0.66	10.29±1.37	10.16±1.55
P value		0.315	0.009*	0.010*	0.010*
				0.028**	

*p value of comparison between control +ve and other study groups by unpaired t- test,
**p value of comparison among six groups (apart from control) by ANOVA.

Effect of latanoprost (0.005%) Drop (Response of mean intraocular pressure)

Post induction of ocular hypertension, the mean IOP was (33.1 ± 2.88mmHg). After one hour of latanoprost 0.005% application the mean IOP decreased by (5.19mmHg) with significant effect compared to distilled water that the decrease started from the first day of treatment till the last 7th day with highly significant decrease (p<0.001) (Fig 2).

Effect of Atorvastatin (0.5%, 1%,2%) Ophthalmic drop (Response of mean intraocular pressure)

Mean IOP of Atorvastatin *(0.5%):* Post induction of Ocular hypertension, the mean IOP was (32.45±3.07mmHg). After one hour of tested drug instillation the mean IOP decrease by (1.32 mmHg) which was significant (p<0.05), after four days of atorvastatin (0.5%) instillation the mean IOP reduced by (2.26mmHg) but latanoprost 0.005% reduce IOP by (12.48mmHg) that was highly significant (p<0.001) than atorvastatin 0.5%.

There was a significant difference in IOP reduction that found in day 2&5 (p<0.05) and highly significant reduction at day 6 &7 (p<0.001) when compared with distilled Water (Fig 3).

Mean IOP of Atorvastatin (1%): Post induction ,the mean IOP was (30.72±0.91), after one hour of tested drug instillation the mean IOP reduced by(2.67 mmHg).maximum reduction in mean IOP achieved in day 7th(16.84±.92mmHg) that was highly significant (p<0.001)when compared with distilled water along the trial period (Fig 4).

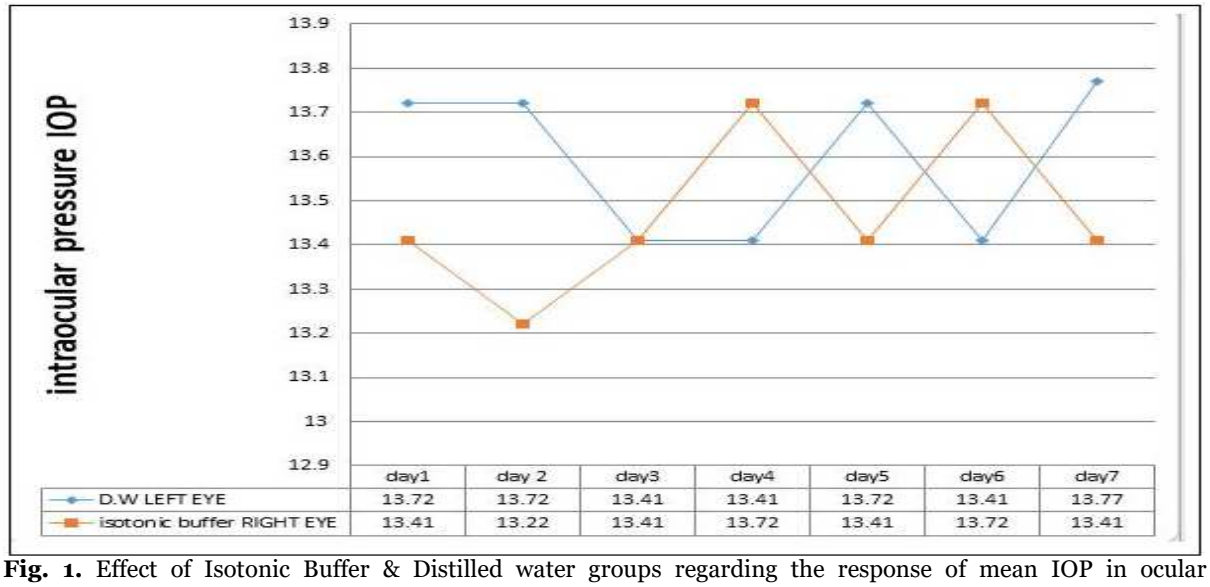


Fig. 1. Effect of Isotonic Buffer & Distilled water groups regarding the response of mean IOP in ocular normotensive rabbits.

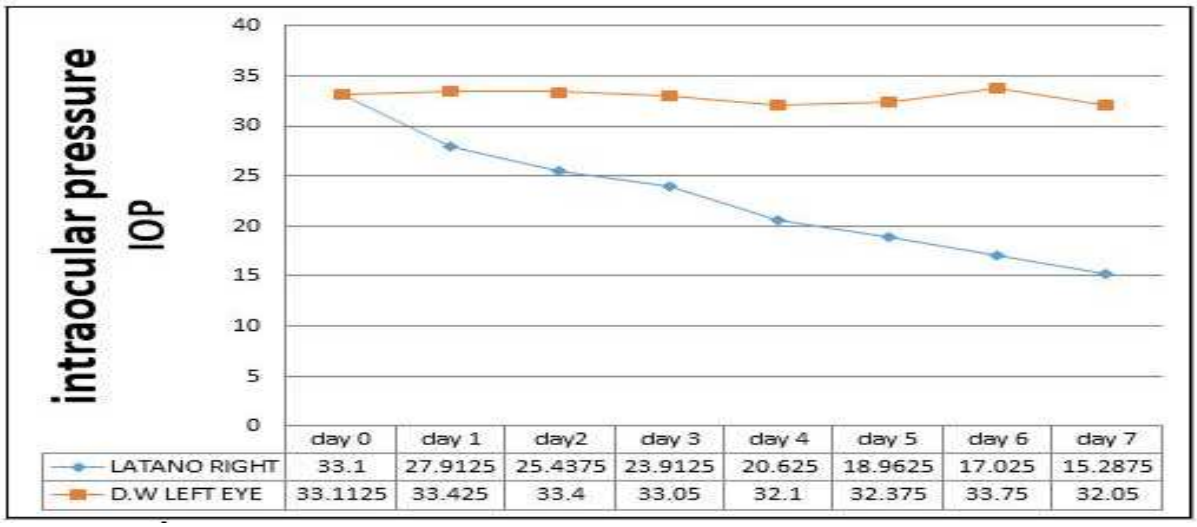


Fig. 2. Effect of latanoprost (0.005%) and DW on mean IOP of ocular hypertensive rabbits.

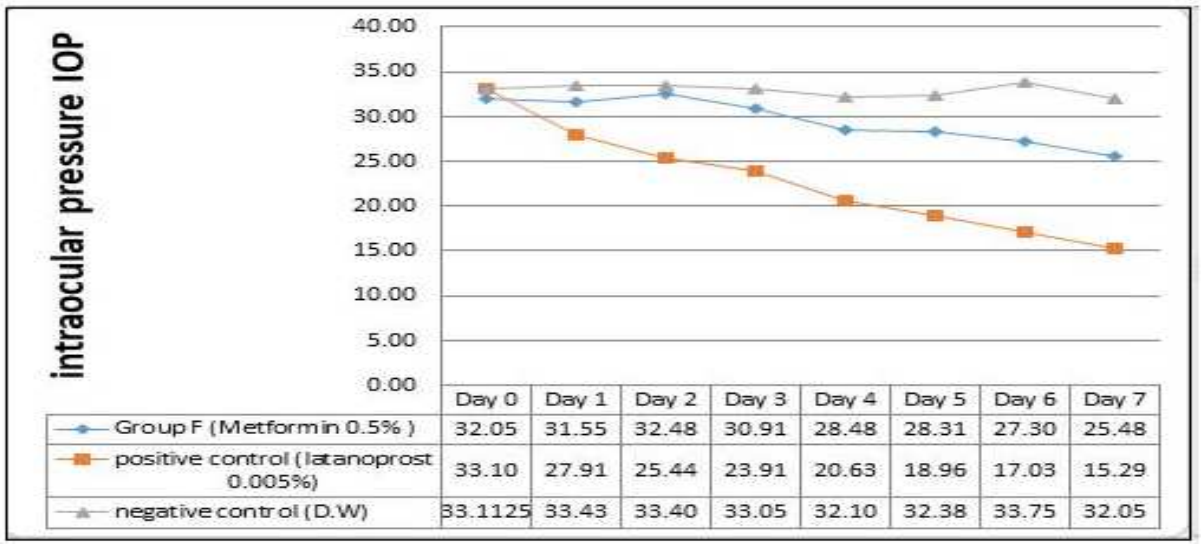
Mean IOP of Atorvastatin (2%): Post induction of ocular hypertension, the mean IOP was (30.13±1.59mmHg). After one hour of atorvastatin (2%) instillation the mean IOP reduced by (4 mmHg), which found to be significant (p<0.05) when compared with latanoprost 0.005%. in the 4th and the 5th, maximum reduction in the mean IOP compared with latanoprost 0.005% that was found to be highly significant (p<0.001) (Fig 5).

Percent of intraocular pressure reduction

Comparing to latanoprost 0.005% IOP reducing effect, atorvastatin 2%, 1% and 0.5% showed the higher IOP reducing effect in the trial study. Figure 6.

Dose response curve of atorvastatin

The figure 7 below shows 0.09 g/L of atorvastatin required to produce 50% decrease in intraocular pressure.



	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Group F (Metformin 0.5%)	32.05	31.55	32.48	30.91	28.48	28.31	27.30	25.48
positive control (latanoprost 0.005%)	33.10	27.91	25.44	23.91	20.63	18.96	17.03	15.29
negative control (D.W)	33.1125	33.43	33.40	33.05	32.10	32.38	33.75	32.05

Fig. 3. Comparison between the effect of atorvastatin (0.5%), latanoprost 0.005% & DW on mean IOP of ocular hypertensive rabbits n=8.

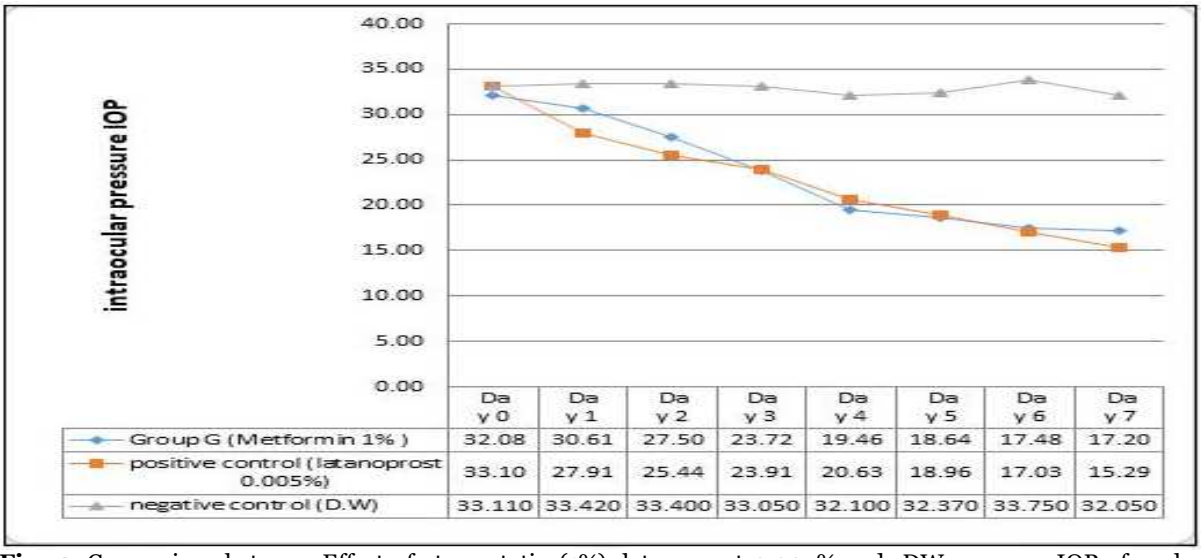
y=-22.45x-3.0067

When y= 50

x= 0.09 g/L required to produce 50% decrease in intraocular pressure.

Glutathione (GSH) levels in rabbit's aqueous humor

There was anon significant elevation of the GSH levels in atorvastatin 0.5% treated group (9.1±7.18Mg/ml) during the period of the treatment. There was a significant elevation ($p<0.05$) of GSH levels in atorvastatin 1% treated group (13.91±7.6Mg/ml).



	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Group G (Metformin 1%)	32.08	30.61	27.50	23.72	19.46	18.64	17.48	17.20
positive control (latanoprost 0.005%)	33.10	27.91	25.44	23.91	20.63	18.96	17.03	15.29
negative control (D.W)	33.110	33.420	33.400	33.050	32.100	32.370	33.750	32.050

Fig. 4. Comparison between Effect of atorvastatin (1%), latanoprost 0.005% and DW on mean IOP of ocular hypertensive rabbits n=8.

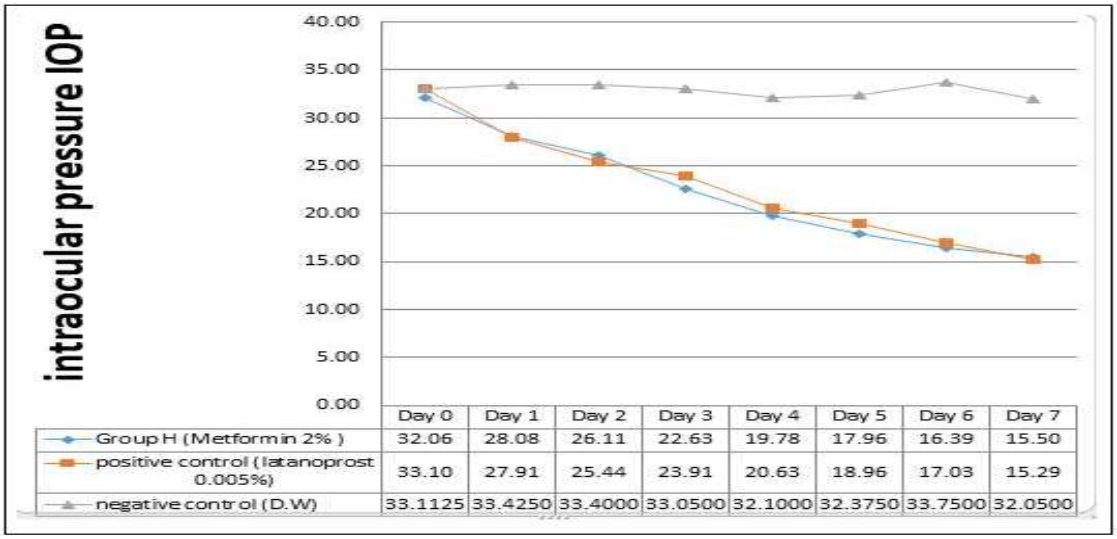
There was a high significant elevation *($p<0.001$)* of GSH levels in atorvastatin 2% treated group (14.42±2.11Mg/ml) (Table 1).

Tumor necrosis factor -alpha (TNF-α) levels in rabbit's aqueous humor

The result show that there was reduction in tumor necrosis factor alpha levels with significant difference of atorvastatin 0.5%, 1%and 2%.when compared with +ve control.

Discussion

In the present study, the distilled water could not change the mean of IOP in normotensive eyes of the rabbits after 7 days of instillation, and there was no significant effect (P > 0.05) when compared mean of IOP during trial period with pre-treatment mean of IOP. This agreed with Heigl *et al.,* (2002).



	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Group H (Metformin 2%)	32.06	28.08	26.11	22.63	19.78	17.96	16.39	15.50
positive control (latanoprost 0.005%)	33.10	27.91	25.44	23.91	20.63	18.96	17.03	15.29
negative control (D.W)	33.1125	33.4250	33.4000	33.0500	32.1000	32.3750	33.7500	32.0500

Fig. 5. Effect of atorvastatin (2%) ,latanoprost 0.005% and DW on mean IOP of ocular hypertensive rabbits n=8.

The eye drops formulation of tested drugs (atorvastatin) were used inactive ingredients which include: sodium chloride, benzalkonium chloride, ethanol, and phosphate buffer. Furthermore, these inactive ingredients did not changed the mean of IOP in normotensive eyes of the rabbits eyes after 7 days of inactive ingredients instillation and there was no significant effect (p>0.05) when compared the mean of IOP during pre-treatment mean of IOP with trial period, this agreed with Allen and Popvich, (2005).

The effectiveness of betamethasone as an inducing agent for ocular hypertension could not counteract by the distilled water. Furthermore, distilled water had no effect on the tested parameters in this study thus; it could be accepted as a negative control group regards the study of effect of tested drugs. This effect confirmed by Urcola *et al*, (2002).

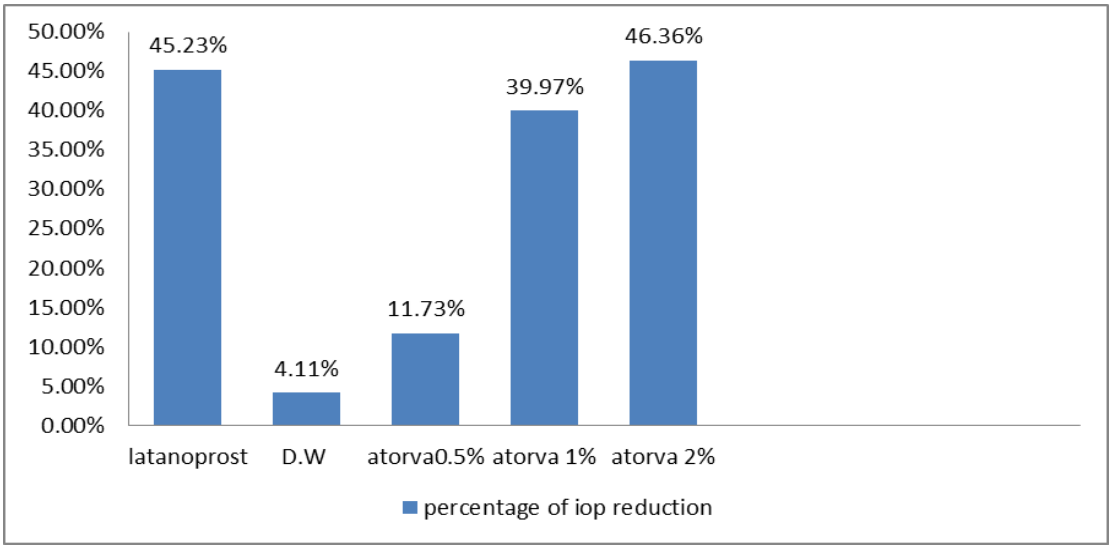


Fig. 6. Percentage of IOP reduction of atorvastatin during the trial period.

The latanoprost had a noticeable ocular hypotensive effect on induced hypertensive eyes. Latanoprost (0.005%) eye drop was used as a positive control to test the ocular hypotensive effect of the most experimented drugs and its preferred in chronic open glaucoma (Achilles and Pappano ,2007). Latanoprost (0.005%) produced (45.23%) reduction in mean IOP at day 7, these results agreed with previous studies (Patelska *et al*,1997; Perry *et al,* 2003; Gupta and yucel, 2007). Latanoprost 0.005% drop application in the present study had no effect on pupil diameter and no effect regarding other possible side effect (i.e. light reflex, corneal reflex, conjunctival redness and lacrimation).

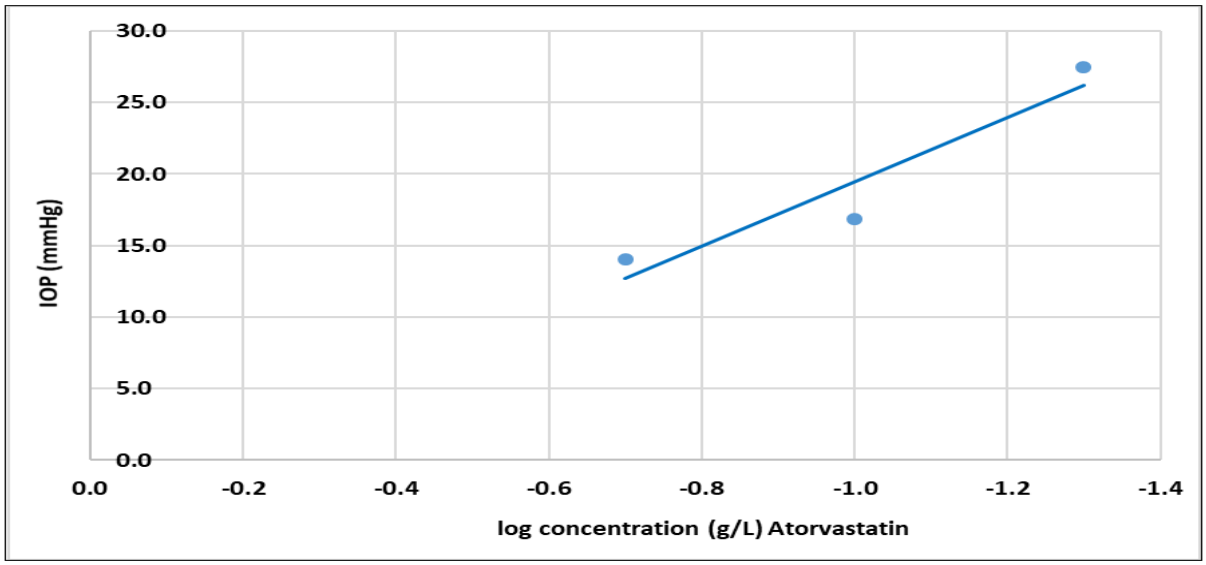


Fig. 7. Dose response curve of Atorvastatin.

The present study demonstrated that single drop of atorvastatin *0.5%* produce an obvious hypotensive effect and reduce the mean IOP by (11.73%) after 7 day treatment when compared with that of DW group, and there is a comparable hypotensive effect when we compared atorvastatin (0.5%) to that of latanoprost group. Also the present study clearly demonstrated that atorvastatin (1%) was able to decrease mean IOP in ocular hypertensive rabbits by (39.97%) after 7 day treatment. Also the results demonstrated that atorvastatin (2%) was able to reduce the IOP by (46.36%) after 7 days treatment. Atorvastatin drop application had no effect on pupil diameter and no effect regarding other possible side effect (i.e. light reflex, corneal reflex, conjunctival redness and lacrimation).

Regarding the anti-inflammatory and the anti-oxidant parameters of atorvastatin, results demonstrated that topical instillation of atorvastatin (0.5%, 1%, 2%) produce a significant elevation in glutathione (GSH) levels, and a significant reduction in tumor necrosis factor –alpha levels in rabbits aqueous humor.

Conclusion

The topical instillation of atorvastatin have a significant intraocular pressure lowering effect as compared with negative control group (DW group) and positive control group (Latanoprost group). and have a significant antioxidant activity) and anti-inflammatory activity also it was found to be relatively Safe in their applied doses .

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