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Original Article

Topical Losartan Reduces IOP by Altering TM Morphology in Rats with Steroid-induced Ocular Hypertension

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Abstract

Purpose: Elevated intraocular pressure (IOP) in primary open angle glaucoma patients is associated with extracellular matrix (ECM) remodeling in trabecular meshwork (TM). Prevention of ECM remodeling may be of great benefit in attenuating long-term deterioration of TM morphology. Since, inhibitors of renin-angiotensin system are known to prevent ECM remodeling in tissues such as heart, we investigated if the IOP lowering effect of angiotensin receptor blocker, losartan, is associated with altered ECM remodeling in TM.

Methods: Effect of single drop and 3-week long multiple drop application of losartan potassium 2% on IOP was evaluated in steroid-induced oculohypertensive rats. Secondly, the effect of topical losartan on aqueous humor matrix metalloproteinase (MMP) -2 and -9 levels and TM morphology was studied. A comparison was made with latanoprost 0.005%.

Results: Single drop treatment with losartan resulted in 26.86% reduction in IOP from baseline, 8 hours post-instillation. The peak IOP lowering effect of losartan was comparable to latanoprost, however, the duration of this effect was 12 hours compared to 24 hours with latanoprost. Twice daily instillation of losartan over 3 weeks caused sustained IOP lowering which was comparable to once daily latanoprost. The IOP lowering effect of losartan was associated with significantly elevated aqueous humor MMP-2 and -9 levels, significantly increased TM cellularity and significantly reduced TM thickness compared to vehicle treated rats.

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Conclusions: Topical application of losartan reduces IOP in steroid-induced ocular hypertensive rats due to alteration in TM tissue remodeling, which could be attributed to increased MMP-2 and -9 secretion in the aqueous humor.

Introduction

Glaucoma, the leading cause of irreversible blindness, is often associated with elevated intraocular pressure (IOP). IOP is determined by the critical balance between the rate of secretion of aqueous humor and rate of its outflow mainly through trabecular meshwork (TM) and Schlemm's canal and to a little extent through uveoscleral pathway. Elevated IOP in patients with primary open angle glaucoma (POAG) is often attributed to increased resistance in TM due to increased deposition of extracellular matrix (ECM). The ECM in TM undergoes continuous remodeling by matrix metalloproteinases (MMPs), which are constitutively secreted by TM cells (Alexander et al. 1991; Bradley et al. 2003; Kelley et al. 2007). Reduced MMP levels favour deposition of ECM and decreased MMP levels have been reported in glaucomatous eyes (Nga et al. 2014; Määttä et al. 2006). IOP elevation in steroid-induced glaucoma is also associated with increased ECM deposition in TM (Razali et al. 2015a; Razali et al. 2015b; Razali et al. 2016). Snyder et al. (1993) have shown that corticosteroid treatment of TM organ and cell cultures causes increased ECM deposition. Hence, steroid-induced glaucoma in animals is considered a close representation of TM changes in glaucomatous human eyes (Agarwal and Agarwal 2017).

Several drug classes are currently used in clinical practice and majority of these drugs reduce IOP by improving the aqueous outflow facility. IOP reduction, however, is often suboptimal and is associated with several adverse effects. Furthermore, most of the current medications are not known to prevent TM remodeling. The ability of drugs to prevent TM remodeling could be of great significance particularly in view of controlling or preventing underlying pathological changes and hence providing long term control of IOP. Prostaglandin analogs such as latanoprost have been studied for their possible effects on MMPs and tissue inhibitors of metalloproteinases (TIMPs) in the TM. However, the studies have not shown a significant effect of this class of drugs on MMPs (el-Shabrawi et al. 2000; Oh et al. 2006; Pradhan et al. 2015). Over the past decade inhibitors of renin–angiotensin system (RAS) have become the drugs of interest as studies have shown the IOP lowering and neuroprotective effects of inhibitors of RAS (Shah et al. 2000; Costagliola et al. 2000; Yang et al. 2009; Mehta et al. 2010; Loftsson et al. 2010). Several components of RAS have been detected in ocular tissue (Vaajanen and Vapaatalo 2011). Our previous studies also showed the IOP lowering effects of angiotensin converting enzyme inhibitor (ACEI), enalaprilat, and angiotensin receptor blocker (ARB), losartan, in oculonormotensive rats (Agarwal et al. 2013). Although, IOP lowering effects of inhibitors of RAS are widely known in human and animals, it remains unclear if the IOP lowering effect of inhibitors of RAS involves increased MMP secretion and prevention of TM remodeling.

Hence, for the first time, we investigated the effects of 3 week long topical administration of losartan on aqueous humor MMP levels and TM morphology and evaluated the association of these changes with those in the IOP of rats with steroid-induced ocular hypertension.

Methods

Sprague Dawley rats weighing 100-120 g of either sex were housed under standard laboratory conditions of 12-hour light/12-hour dark cycle and free access to food and water. All the methods used in this study complied with ARVO statement of Use of Animals in Ophthalmic and Vision Research. Losartan potassium (ChemoLab) 2% solution was prepared in HPMC 1%.

Commercially available Latanoprost 0.005% (Xalatan®) was used as reference standard. HPMC1% was used as vehicle.

IOP estimation: IOP was estimated using a calibrated tonometer (TonoLab, Icare®, Finland) in conscious animals without topical anesthesia. Before starting the experiment, ten normotensive rats weighing 120-140 g were subjected to IOP measurements using TonoLab by two independent observers, twice at one week interval. Each observer was blinded to the IOP recorded by the other observer and to their own recordings on previous occasion. All IOP estimations were carried out between 8.00 and 10.00 AM. The mean value of 6 consecutive readings at all time points was considered as final estimation. Subsequently, mean intraobserver and interobserver differences were calculated.

Experimental model: The steroid-induced model of ocular hypertension was developed as described previously (Razali et al. 2015b). Briefly, after recording the baseline IOP, rats were treated bilaterally and topically with 5 µl of dexamethasone 0.1%, twice a day for 36 days. The IOP was measured twice a week during 36 days of treatment. At the end of 36 days, rats with IOP elevation of at least 25% above baseline were considered to be oculohypertensive and were included in the study.

Study 1: The oculohypertensive rats were randomly divided into 2 groups of 6 animals each. After estimation of baseline IOP, the animals in group 1 were topically treated with losartan 2% in one of the randomly chosen eyes (Test eye). Similarly, group 2 received latanoprost 0.005% in the test eye. The contralateral eyes in both the groups received single drop of vehicle. Single drops of vehicle as well as losartan/latanoprost were of 5 µl volume. After instillation of drug/vehicle, IOP was measured at regular intervals using Tonolab.

Study 2: The oculohypertensive rats were randomly divided into 3 groups of 6 animals each. After estimation of baseline IOP, animals in one of the groups received single drop of vehicle in both eyes. The 2 other groups were bilaterally treated with losartan or latanoprost. Losartan 2% was applied topically twice daily, while the latanoprost 0.005% was administered once daily. Another group consisted of normotensive rats that received treatment with HPMC 1% for 36 days instead of dexamethasone. Bilateral topical administration of respective drugs or vehicle in all groups was done daily in a volume of 5 µl for a total of 21 days. IOP was estimated twice a week between 8.00 and 10.00 AM and the drug instillation in the morning was done after IOP measurement to allow assessment of IOP reduction corresponding to trough concentrations of drugs.

At the end of three weeks treatment period, the aqueous humor was collected before the rats were sacrificed. The rats were anaesthetized by subcutaneous injection of ketamine and xylazine solution. Once the rats were unconscious, the eyeballs were washed using phosphate buffer saline (PBS) and wiped clean. The cornea of the eye was then pricked by a sterile needle. The aqueous humor was immediately collected using a 20-µl micropipette into a siliconized microcentrifuge tube. The aqueous humor sample was stored at –80°C till further processing for estimation of MMP levels. Pooled aqueous humor from 2 eyes of same animals was used as one sample. Commercially available Rat MMP-2 and MMP-9 Elisa Kits (Abnova, Taiwan) were used to quantify MMP-2 and MMP-9 in the aqueous humor.

After aqueous humor drainage, rats were sacrificed by overdose of ketamine and xylazine, the eyes were enucleated and the eyeballs were fixed in 10% formalin solution. The enucleated eyes were paraffin embedded, sectioned and subjected to hematoxylin and eosin staining for histopathological examination of TM.

Statistical methods

All data is presented as Mean±SD. Comparison of IOP between two eyes in the same group was done using paired student t-test. Intergroup comparisons for study 2 were done using one-way Anova. $P<0.05$ was considered significant.

Results

Firstly, IOP lowering effect of unilateral single drop application of losartan potassium was studied using a calibrated tonometer (TonoLab, Icare®, Finland). Subsequently, effect of bilateral topical application of losartan over 3 weeks on IOP, aqueous humor MMP-2 and -9 levels and TM morphology was studied. For both studies Sprague Dawley rats were used in which ocular hypertension was induced by topical application of dexamethasone for 36 days.

IOP measurements and effect of dexamethasone of IOP

Before starting the experiment, technique of IOP measurement was standardized to ensure repeatability and reproducibility of the method. The IOP estimation by first observer was 13.4±1.27 and 13.35±1.27 mmHg on the first and second occasion, respectively. Mean IOP estimation by second observer was 13.7±1.26 and 13.75±1.37 mmHg on first and second occasion, respectively. The mean differences were 0.05±1.10 and 0.05±1.28 mmHg for first observer and second observer respectively. All intraobserver and interobserver differences of IOP estimation were within 2 SD from mean, which indicates good repeatability and reproducibility of IOP estimation by Tonolab.

After starting the topical treatment with dexamethasone, IOP elevation in both eyes was observed from day 8 until day 36 ($p<0.05$). The IOP reached plateau after 32 days. There was no significant difference between the mean IOP of right and left eyes at any time point. At the end of 36 days, the mean IOP elevation achieved was 42.29 and 46.13% for right and left eyes respectively. Fig. 1 and 2 show the progressive increase in IOP from baseline over 36 days of steroid treatment.

IOP lowering effect of single drop application of losartan potassium versus latanoprost

Topical treatment with single drop of losartan caused significant reduction of IOP compared to baseline starting from the first hour until the 10th hour post-treatment. Whereas in latanoprost treated group, IOP reduction started 3 hour post-instillation and lasted until 24th hour post-treatment. Maximum mean IOP reduction of 26.86% was observed at 8 hour post-instillation in losartan treated group but the same was 28.40% at 10th hour post-instillation in latanoprost group. The maximum IOP reduction by losartan potassium was comparable to latanaprost ($p>0.05$). However, it was observed that the duration of significant IOP reduction in losartan group was 10 hours compared to 24 hours in latanaprost group (Table I, Fig. 1).

TABLE I: IOP lowering effect of single drop application of losartan potassium 2% versus latanoprost 0.005% in rats with steroid–induced ocular hypertension. All values represent group mean.

Time post–instillation (hour)	*Losartan % of reduction from baseline*	*Latanoprost % of reduction from baseline*
0	–	–
1	–5.07	0.36
2	–9.49	–4.31
3	–15.53	–4.48
4	–17.58	–8.62
6	–20.82	–13.01
8	–26.86	–17.80
10	–13.59	–28.40
12	–3.02	–15.49
16	0.22	–5.47
20	–0.65	–5.47
24	–0.43	–4.39

IOP lowering effect of bilateral multiple drop application of losartan potassium versus latanoprost over 3 weeks

Bilateral treatment with losartan significantly ($p<0.01$) reduced IOP of steroid-induced oculohypertensive rats compared to vehicle treated oculohypertensive rats. The reduction in IOP was sustained over the treatment period without significant fluctuations. A similar observation was made in latanoprost treated group (Table II, Fig. 2).

Effect of losartan on aqueous humor concentration of MMP-2 and MMP-9

MMP-2 and -9 concentrations in oculohypertensive vehicle treated group were 6.29 and 2.10 fold lower, respectively, compared to normotensive group. In losartan treated group, there was 3.32 and 1.89 fold increase in the concentration of MMP-2 and -9,

TABLE II: IOP lowering effect of daily topical application of losartan 2% versus latanoprost 0.005% over 3 weeks in rats with steroid-induced ocular hypertension. All values represent group mean.

Time (Days post–treatment)	*IOP Reduction from baseline (%) after 36 days of steroid/vehicle treatment*							
	Normotensive		*Oculohypertensive*					
			Vehicle		*Losartan 2%*		*Latanoprost 0.005%*	
	Right eye	*Left eye*	*Right eye*	*Left eye*	*Right eye*	*Left eye*	*Right eye*	*Left eye*
3	–0.35	0.10	–0.67	–0.88	–14	–14	–22.76	–24.99
7	–0.89	–0.25	0.40	0.38	–15	–14	–20.41	–21.44
11	–1.33	–0.94	–0.98	–1.70	–18	–17	–21.86	–21.79
14	–0.89	0.35	–0.41	–0.31	–17	–16	–29.61	–29.66
18	–0.80	0.67	–1.10	–1.07	–21	–21	–29.34	–29.38
21	–2.04	0.87	–0.81	–0.63	–19	–18	–21.90	–23.65

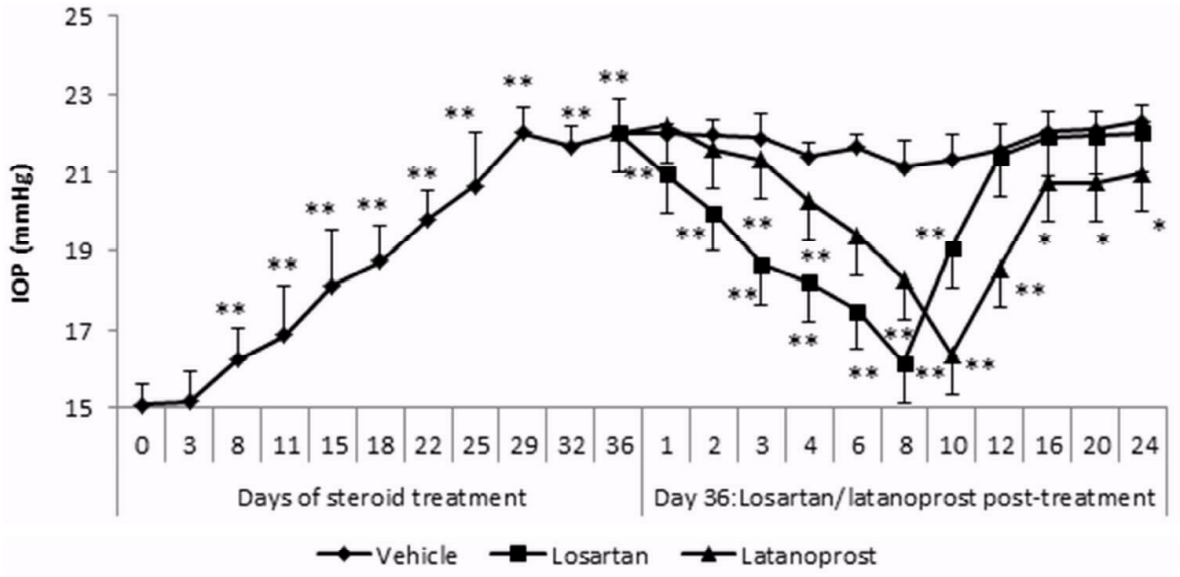

Fig. 1: Effect of unilateral single drop application of losartan 2% after 36 days of treatment with dexamethasone in rats. Up to day 36 (Days of steroid treatment): *p<0.05; * *p<0.01 vs. corresponding baseline. On Day 36: *p<0.05; * *p<0.01 test eye vs. control eye. Since control eyes of both the latanoprost and losartan treated groups showed no differences at any time point, single data series from control eye is shown on day 36.

respectively, compared to oculohypertensive vehicle treated group. Although MMP-2 concentration in losartan treated group remained lower than normotensive group ($p<0.05$), both MMP-2 and -9 levels were higher in this group compared to latanoprost treated group ($p<0.01$ and <0.05, respectively). In latanoprost treated group, MMP-2 levels were higher than oculohypertensive vehicle treated group ($p<0.05$) but no difference was observed for MMP-9 (Table III).

Effect of losartan on TM morphology

The H&E stained sections were examined for TM morphology. Thickness of TM in four groups was measured at 4 sites as shown in Figure 3A. The average of 4 measurements was taken as the final estimate. The vehicle treated oculohypertensive rats showed significantly greater mean TM thickness compared to normotensive group ($p<0.01$). Losartan-treated group showed significantly lower ($p<0.01$) TM thickness compared to the vehicle-treated oculohypertensive rats. Latanoprost treated group also showed a significantly thinner ($p<0.01$) TM compared to the oculohypertensive vehicle-treated group (Table IV).

We also did a cell count in the 100 µm

TABLE III : Mean aqueous humor MMP-2 and -9 concentrations among 4 groups.

	Normotensive	*Oculohypertensive*		
		Vehicle	*Losartan 2%*	*Latanoprost 0.005%*
MMP-2 (ng/ml) (N=6)	21.85±4.92	3.47±1.33**	11.55±0.60*$^{\#\#\$\$}$	6.34±0.28**$^{\#}$
MMP-9 (pg/ml) (N=6)	12.32±3.50	5.87±0.35*	11.09±2.46$^{\#\#\$}$	6.99±2.14*

All values are expressed as Mean±SD. *(p<0.05); **(p<0.01) vs. normotensive group, $^{\#}$(p<0.05); $^{\#\#}$(p<0.001) vs oculohypertensive vehicle treated group. $^{\$}$p<0.05; $^{\$\$}$p<0.01 versus oculohypertensive latanoprost treated group.

TABLE IV : Mean TM thickness and number of cells among 4 groups.

	Normotensive	*Oculohypertensive*		
		Vehicle	*Losartan 2%*	*Latanoprost 0.005%*
Trabecular meshwork thickness (µm) (N=6)	9.98±1.57	13.62±1.45**	9.29±1.88 $^{\#\#}$	9.53±1.42$^{\#\#}$
Number of Cells (N=6)	23.61±4.63	15.86±6.61***	22.05±6.12$^{\#\#\$}$	18.57±4.53**

All values are expressed as Mean±SD. *p<0.05, **p<0.01, ***p<0.001 vs normotensive group; #p<0.05 and ## p<0.01 vs oculohypertensive vehicle treated group; $^{\$}$p<0.05 vs oculohypertensive latanoprost treated group.

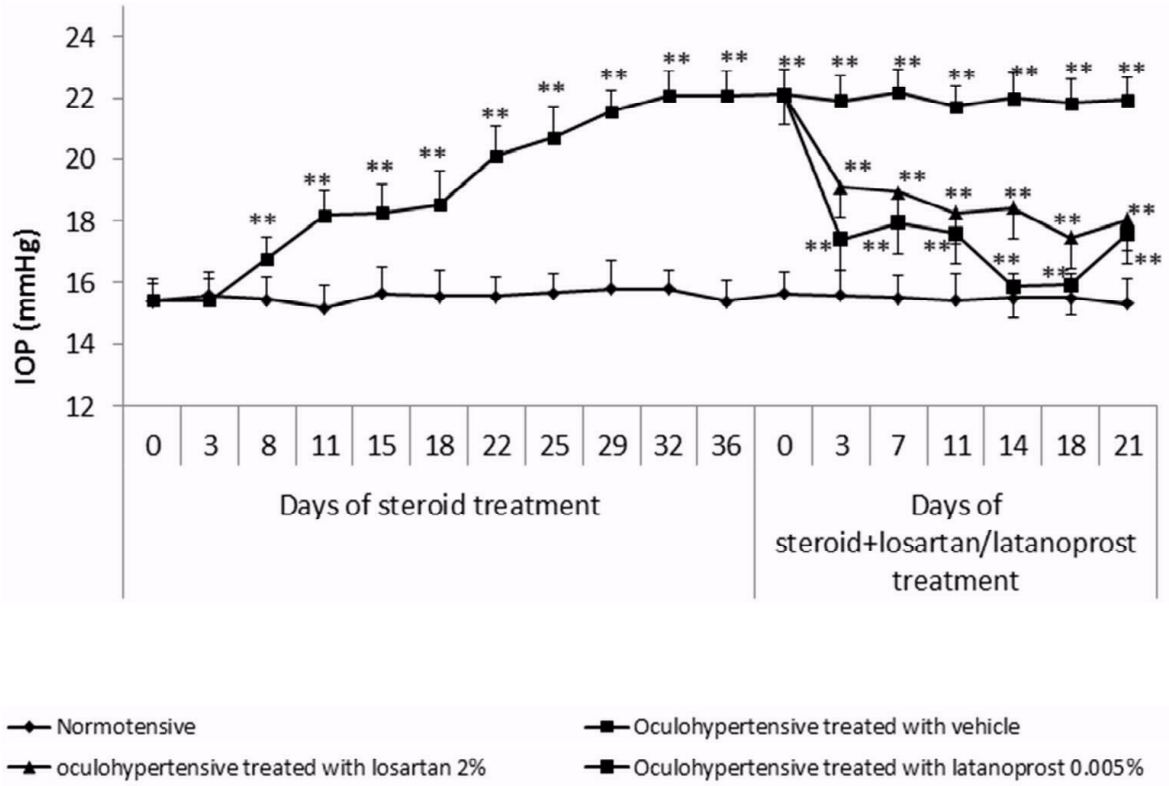

Fig. 2 : Effect of repeated dose application of losartan 2% over 3 weeks in rats with steroid-induced ocular hypertension. Each data series represents average IOP of right and left eyes. Up to day 36 (Days of steroid treatment): **p<0.01 vs. corresponding baseline. From day 0 to day 21 (Days of steroid + losartan/latanoprost treatment): **p<0.01 versus oculohypertensive vehicle treated group.

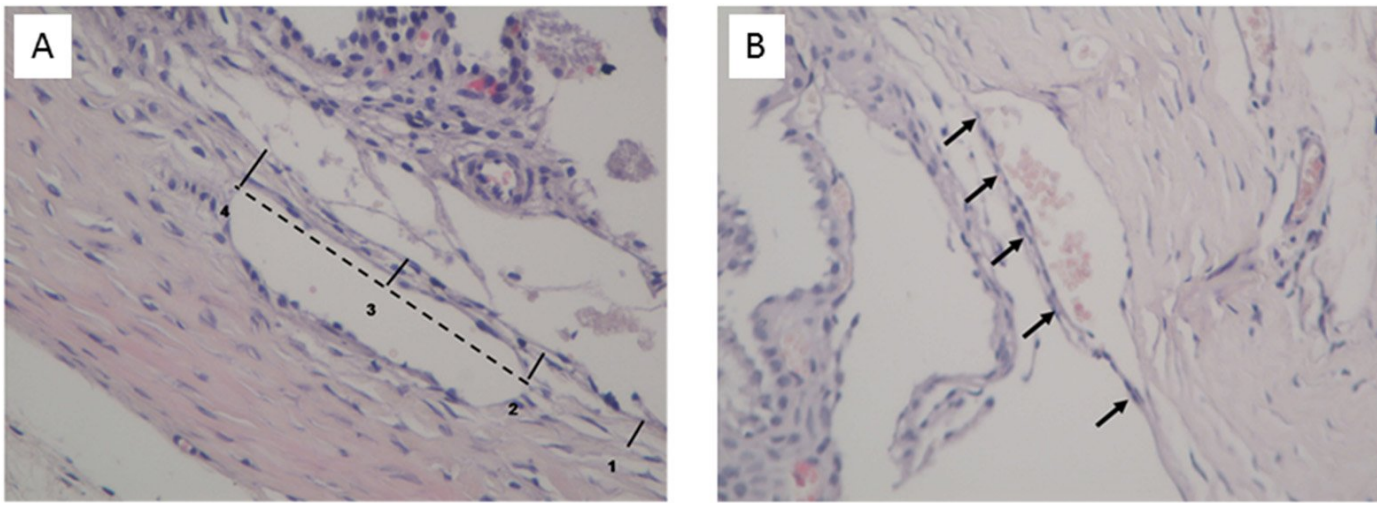

Fig. 3 : 3A: Photomicrograph showing the anterior chamber angle of a normal Sprague–Dawley rat. Lines 1–4 indicate the positions at which TM thickness was measured. 3B: The cells were counted in the 100 µm paracanalicular area as indicated by arrows.

paracanalicular area as indicated in Fig. 3B. The spindle shaped nuclei of fibrous connective tissue cells were counted in the predefined trabecular area as indicated in Fig. 3B. There was a significant difference ($p<0.05$) between the number of cells between normotensive and oculohypertensive vehicle-treated group, with significantly higher mean number in normotensive group. The cell count in losartan-treated group was significantly higher than oculohypertensive vehicle-treated as well as latanoprost treated groups and was comparable to that in normotensive rats. In latanoprost-treated group, the cell count remained significantly lower than the normotensive group (Table IV).

An average of both the TM thickness and TM cell count from 2 eyes of each animal was considered as representative of one sample. Both morphological estimations were done by two masked observers independently and the average of two was taken as the final estimate.

Discussion

Systemic RAS is known to play a significant role in blood pressure homeostasis. It is now also considered to play an important role in tissue-specific regulatory functions, hence producing local effects and long-term changes in tissues. Presence of the components of RAS in ocular tissue has been described by several researchers (Danser et al. 1994; Savaskan et al. 2004; Paul et al. 2006; White et al. 2015). Accordingly, besides angiotensin II type 1 receptor blockers, IOP lowering effects of angiotensin converting enzyme inhibitors and Mas receptor agonist have been investigated in few studies (Loftsson et al. 2010; Vaajanen et al. 2008). Among angiotensin receptor blockers, CGP 48933, losartan and CS-088, were shown to reduce IOP when applied topically to rabbit eyes (Kaiser et al. 1997; Shah et al. 2000; Inoue et al. 2001) or CS-088 in monkey eyes (Wang et al. 2005). Kaiser et al. also studied the effects of topical losartan in human, however, no significant effect on IOP was observed. It is noteworthy that this human study involved only 5 patients. None of these studies have described if angiotensin receptor blockers can produce sustained IOP reduction on prolonged administration and particularly so in an animal model such as steroid-induced ocular hypertension that could be considered closest to POAG in human (Agarwal and Agarwal 2017).

The current study showed that the single drop application of losartan produces significant IOP reduction in rats with steroid-induced oculohypertension. The peak IOP lowering by single drop of losartan was comparable to that produced by latanoprost, however, the duration of significant IOP lowering was longer with latanoprost. This study

for the first time has demonstrated that repeated dose administration of losartan over 3 weeks produces sustained IOP reduction which is comparable to that produced by latanoprost. The frequency of administration of losartan, however, was twice daily compared to once daily administration of latanoprost due to its longer duration of action. Since very few studies have been done to investigate the IOP lowering effects of angiotensin receptor blockers, the mechanisms underlying these effects remain unclear.

One of the recent studies has shown that the IOP lowering effect of systemically administered losartan in CD1 mice is associated with prevention of RGC loss (Quigley et al. 2015). Instead of possible vascular effects of losartan in retina leading to neuroprotective effects, this study provides evidence that these effects of losartan are attributed to reduced thickness of sclera which normalized the biomechanical scleral response to elevated IOP. The scleral thinning in response to losartan treatment involved inhibition of increase in pERK and reduced scleral fibroblast activation. Losartan was also shown to produce similar responses in mouse model of Marfan syndrome in the presence of intact angiotensin II signaling (Habashi et al. 2011). Both of these studies demonstrated higher effectiveness of losartan compared to enalaril for the possible reason that losartan maintains or even enhances signaling through angiotensin II type 2 receptors whereas enalapril limits signaling through both angiotensin II type 1 and type 2 receptors. Angiotensin receptor blockers have also been shown to attenuate cardiac remodeling (Zornoff et al. 2000), however, it is not known if similar responses could be observed in TM. Within TM, the major site of increased resistance is the juxtacanalicular tissue (JCT). Continuous remodeling of ECM in JCT is critical in preserving aqueous outflow channels in open state by releasing trapped debris and associated ECM fragments from the outflow pathways (Keller and Acott 2013). Studies have shown that in POAG eyes, there is significant reduction in the number of TM cells and significant increase in the ECM deposition in JCT (Alvarado et al. 1984; Grierson and Howes 1987; Lütjen-Drecoll et al. 1981). TM cells are responsible for detecting mechanical stimulus of elevated IOP and therefore, their reduced number limits the ability of tissue to respond to elevated IOP (Keller and Acott 2013). Similar TM changes have been reported earlier in rat eyes with steroid-induced glaucoma (Tektas and Lütjen-Drecoll 2009). Our previous studies have also shown that treatment of human TM cells with dexamethasone results in increased expression of collagen type I, III, IV, fibronectin and alpha smooth muscle actin (Hassan et al. 2016). Hence, reduced TM thickness indicating reduced ECM deposition and increased TM cellularity observed in the current study clearly demonstrate that losartan positively alters TM tissue composition resulting in IOP lowering.

MMPs have been described as important modulators of aqueous humor outflow as they continuously remodel ECM composition in TM and maintain a stable outflow resistance and IOP (De Groef et al. 2013). The current study for the first time shows that topical treatment with losartan results in increased aqueous humor MMP-2 and -9 levels. In this study we measured total MMP levels and not the active MMPs. However, it could be assumed that even if increased MMP level are due to proMMPs there is larger readily available pool for production of active MMPs after treatment with losartan. In line with our observation, one of the studies showed that the treatment of vascular smooth muscle cells with angiotensin II reduced MMP-2 secretion but treatment with losartan inhibited the effect of angiotensin II (Papakonstantinou et al. 2001). Varo et al. also showed that chronic treatment of spontaneously hypertensive rats with losartan reverses myocardial fibrosis and this effect of losartan involves increased collagenase activity along with reduced expression of TIMP1. Similarly both the short and long-term treatment of rat aortic smooth muscle cells with angiotensin II were shown to increase the TMIP-1 expression (Castoldi et al. 2003). TIMPs are inhibitors of MMPs and hence an altered ratio of MMP/TIMP in response to losartan has possibly contributed to reduced TM thickness in the current study.

It can be concluded from the results of this study that IOP reduction in response to topical losartan in rats with steroid-induced ocular hypertension is associated with increased TM cellularity and reduced

ECM deposition and these morphological changes could be attributed to increased MMP-2 and -9 secretion. It is also noteworthy that transforming growth factor-β is involved in increased ECM deposition in the TM of glaucomatous eyes (Agarwal and Agarwal 2010; Agarwal and Agarwal 2015) and angiotensin II stimulates collagen production via TGF-β-dependent pathways. Hence, inhibitors of RAS may also reduce ECM deposition by inhibiting TGF-β signaling (Diop-Frimpong et al. 2011). Therefore, the effects of losartan on TM morphology observed in the current study may involve not only the pathways that enhance ECM degradation involving increased MMP secretion but also those that inhibit ECM deposition. Additionally, angiotensin II acts as a secretagogue in human ciliary body non-pigmented epithelium and increases aqueous humor secretion via angiotensin II type 1 receptors. Treatment with losartan inhibits this activity of angiotensin II (Cullinane et al. 2002). Hence, reduced rate of aqueous humor secretion may also be a contributory factor in the IOP lowering effect of losartan in the current study. Moreover, the contractile effects of angiotensin II on vascular smooth muscles are countered by losartan and since TM cells possess smooth muscle like properties, losartan may cause TM relaxation, an effect that contributes to reduced outflow pathway resistance (Abu-Hassan et al. 2014).

In conclusion, the current study showed that topical application of losartan lowers IOP. This lowering of IOP is associated with increased cellularity and decreased ECM deposition in TM. The morphological changes in TM in response to losartan could be attributed to increased aqueous humor levels of MMP-2 and -9. Further studies are needed to explore the molecular pathways involved in losartan-induced increase in MMP levels, effects of losartan on pathways involved in ECM biosynthesis in TM and other mechanisms that may contribute to changes in aqueous humor dynamics.

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Competing interests:

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Contributors:

All authors have substantially contributed to conception, designing, drafting the article and in final approval of the manuscript version to be submitted. All authors have jointly decided to designate Assoc Prof Dr Renu Agarwal to be responsible for taking decision regarding the presence of authors and the order of their presence in the manuscript. Assoc Prof Dr Renu Agarwal has also been selected by all authors to be responsible for all future communication with the journal regarding this manuscript.

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