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RESEARCH ARTICLE

Studies on Corneal Permeation and Oculo-Hypotensive Effect of Benazepril in Chronic and Acute Models of Glaucoma

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ABSTRACT

The present study was carried out to investigate the effect of benazepril on corneal permeation in goat cornea and its effect on experimentally induced acute and chronic glaucoma in rabbits. Acute glaucoma was produced by i.v. infusion of 5% glucose (15 mL/kg) in rabbits, whereas chronic glaucoma was induced by injection of alpha-chymotrypsin into posterior chamber of rabbit eye. We studied the interaction of benazepril on isolated rat ileum pre-administered with acetylcholine, the enzyme cholinesterase biochemically and on ACE levels in aqueous humor after topical application. A significant increase in intra ocular pressure (IOP) in rabbits was observed which reached to the peak of 41 ± 0.85 after 90 min. Injection of alpha-chymotrypsin produced sustained elevation of Intraocular Pressure which lasted for almost 3 months. Benazepril produced significant fall in Intraocular Pressure in normotensive as well as glucose or alpha-chymotrypsin treated rabbit eyes. It was been observed that the benazepril produced significant potentiation of responses to acetylcholine in isolated rat ileum preparation and inhibition of cholinesterase enzyme. The corneal permeation of benazepril from 0.1% solution was maximum at 15 min after which there is a decline in rate of permeation was observed. The results observed in the present study indicate that the potential ocular hypotensive activity of benazepril which may be due to inhibition of ACE (Kininase-II) and cholinesterase.

Keywords: *Benazepril, Intraocular pressure (IOP), Corneal permeation, ACE inhibitor*

The corneal stroma is a transparent tissue predominantly comprised of regularly arranged collagen fibrils as well as small proteoglycans and matrix proteins. The proteoglycans, which are associated with the collagen fibrils [1], include keratan sulfate and the chondroitin/dermatan sulfate families. After topical instillation, drugs are absorbed into the inner eye through the cornea or the conjunctiva and sclera. The cornea is the main route of absorption for clinically used ocular drugs [2,3]. The route through conjunctiva and sclera is important mostly for very hydrophilic and large molecules that are not able to penetrate through the corneal barrier [4]. Most clinically used ocular drugs have adequate lipophilicity for corneal absorption, and such properties are sought in the development of new ocular drugs. The permeability of the cornea to drugs is clinically important because it is the major factor determining the efficacy of topically applied preparation. Hence, we also carried out corneal permeation for Benazepril. Permeability studies of corneal drugs are usually performed *in vitro* using isolated goat corneas mounted in modified Franz cells [5].

Benazepril is well established in the treatment of arterial hypertension, heart failure and diabetic and/or hypertensive nephropathy with albuminuria. All ACE inhibitors except benazepril and lisinopril are administered as prodrugs. Lotti and Pawlowski [6] reported that topical administration of enalaprilat produces a decrease in IOP in African green monkeys. It was suggested by them that ocular hypotensive effect of enalaprilat may be due to its ability to prevent breakdown of bradykinin that promotes synthesis of prostaglandins. Prostaglandins are known to reduce IOP by increasing uveoscleral outflow [7, 8, 9]. There are a few reports that indicate the presence of angiotensin converting enzyme (ACE) in aqueous humor and in various other ocular tissues in different species including humans [10, 11]. Gene expression of renin, angiotensinogen and ACE in various parts of the human eye has been demonstrated [12]. Oculohypotensive effects of ramiprilate, enalapril, fosi-

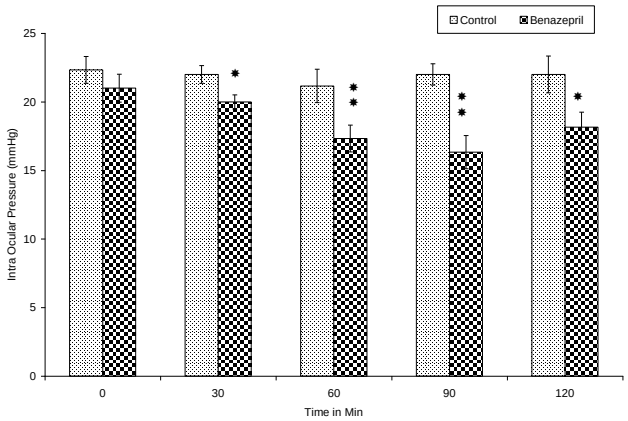

Fig 1. Effect of benazepril on normotensive rabbit eye. * $p < 0.01$, ** $p < 0.001$ compare with control (n=6).

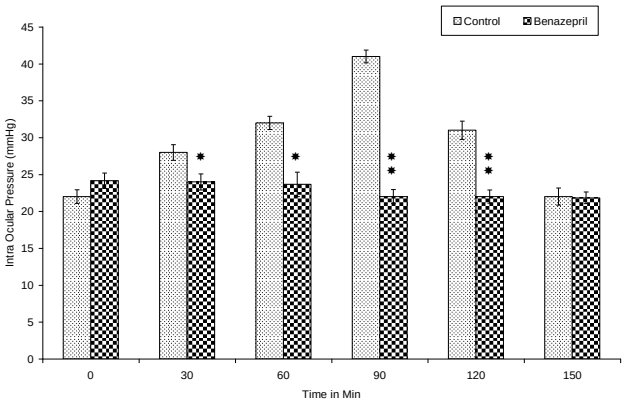

Fig 2. Effect of benazepril in acute glaucoma model. * $p < 0.001$, ** $p < 0.0001$ compare with control (n=6).

nopril have been reported in chronic and acute models of glaucoma [13, 14].

ACE inhibitors have also been reported to interfere with autonomic nervous system and produce various effects such as facilitation of vagal bradycardia [15], inhibition of response to sympathetic spinal outflow [6] and inhibition of vasoconstrictor response to noradrenaline [17]. Several agents that cause inhibition of the enzyme cholinesterase, antagonism of beta-adrenoceptors and stimulation of muscarinic receptors are commonly used as ocular hypotensive agents in glaucoma. The present investigation was undertaken to study the effects of benazepril in acute and chronic models of glaucoma. Attempts were also made to find out the mechanism of action.

MATERIALS AND METHODS

Benazepril was received as gift sample from Wockhardt Research Center, Aurangabad. Alpha-chymotrypsin and Hippuryl–L-histidyl–L–leucine were obtained from Siga, USA. The cholinesterase enzyme kit was procured from Bayer's Diagnostics, Baroda. Drug Acetylcholine, 5% Dextrose i.v. fluid, Ketamine injection and Lignocaine eye drops were obtained from E. Merck Ltd, Mumbai, Bexter Pharmaceuticals, Aurangabad, and Neon pharmaceuticals, Ahmedabad respectively. Newzeland rabbits and Albino Wistar rats obtained from CCS Haryana Agricultural University were maintained in the animal house of our department as per the guideline of CPCSEA. They had access to food and water *ad libitum*. The permission to conduct experiments was obtained from Institutional Animal Ethical Committee (IAEC) of CPCSEA.

IOP in Normotensive Rabbit Eye

Rabbits weighing 1.5 to 2.0 kg were anesthetized with ketamine (50 mg/kg i.v.). Lignocaine (4%) was applied topically to block corneal reflexes. A 30-gauge needle, connected to a pressure gauge transducer, was passed through the cornea into anterior chamber. The change in IOP was recorded continuously using a polygraph (Biodevices, India). After allowing initial stabilization for 10 min, the normal IOP was recorded for both eyes. Freshly prepared benazepril (0.1 mL, 0.1%) in sterile saline solution was administered topically in the left eye. The right eye received only sterile saline and served as control.

Acute Model of Glaucoma

The albino rabbits of either sex weighing 1.5-2.0 kg were infused with 5% glucose solution (15 mL/kg) through marginal ear vein. Half an hour prior to this infusion benazepril (0.1 % W/V) in sterile saline was administered topically to the left eye. Right eye, which received equivalent amount of saline, served as control. Change in IOP was measured every 15 min up to 75 min by a Schiotz type indentation tonometer using 7.5 and 10 g weight. The tonometer used was calibrated for rabbits by open manometric calibration.

Chronic Model of Glaucoma

The technique used to induce model of glaucoma was a modification of that described by Sears and Sears [18]. Rabbits, anaesthetized with ketamine (50 mg/kg i.v.) were used for this experiment. A cannula, attached to a reservoir, was inserted into the anterior chamber with the help of 30 gauge needle, to provide a hydrostatic pressure of 25 mmHg during injection of alpha-chymotrypsin. Then a second cannula appropriately shaped 30 gauge, was introduced near the limbus and directed into the posterior chamber through the pupil. Freshly prepared 150 Units of alpha-chymotrypsin (Sigma, USA) prepared in 0.1 mL of sterile saline, was irrigated through the cannula into the posterior chamber. Care was taken to prevent the contact of alpha-chymotrypsin with corneal stroma. Both the cannula was carefully removed without significant loss of aqueous humor. After two days the intraocular pressure was measured using Schiotz indentation tonometer applying 7.5 and 10 g weight. By drawing a graph of day's against IOP the maximum period required to achieve stable rise in IOP was determined. In our experiments it was found that 15 days were sufficient to achieve stable rise in IOP. Hence, the drug effects on IOP were measured after 15 days for 3 consecutive days every morning, to assure stable IOP. Those rabbits which showed IOP less than 30.0 mmHg were rejected from the study.

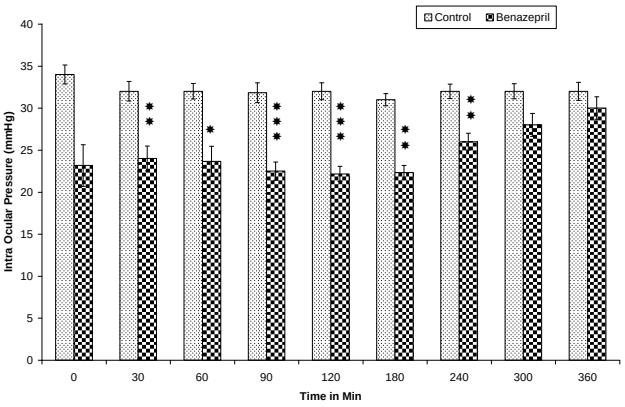

Fig 3. Effect of benazepril in chronic model of glaucoma. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ compare with control (n=6)

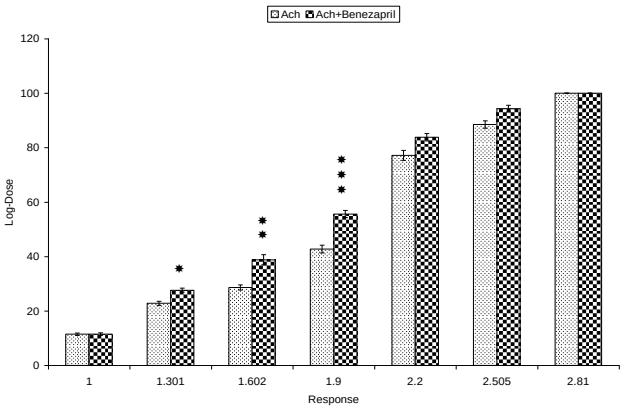

Fig 4. Effect of benazepril on drug response curve of acetylcholine. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ compare with response of acetylcholine (n=6)

After achieving a steady elevated IOP, 0.1 mL of benazepril (0.1%) was administered topically into the left eye whereas, right eye served as control. The drug solutions were prepared in sterile saline. The pressure recordings were carried out at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min after drug instillation.

Studies on Rat Ileum

Male albino rats of Wistar strain weighing 150-200 g were sacrificed by stunning and cutting of neck blood vessels. Ileum was isolated and mounted in organ bath containing tyrode solution which was continuously bubbled with air. The responses to acetylcholine were recorded on a polygraph using force displacement transducer. A basal tension of 500mg. was applied on the ileum. The preparation was stabilized for 30 min. before addition of drugs. Later it was exposed to graded doses of acetylcholine (5.50×10^{-8} to 1.65×10^{-5} M). The contact time for each dose was 45 sec. After taking complete dose response curve, preparations were bathed in solution containing benazepril (8.72×10^{-6} M). After 5 min. the dose response curve of acetylcholine was re-elicited.

Biochemical Estimation of Cholinesterase Enzyme

The effect of benazepril was studied on cholinesterase enzyme *in vitro* in the rabbit blood as per a method recommended by Knedel [19, 20]. Blood was collected through the marginal ear vein, from healthy albino rabbits weighing 2.0–2.5 kg Plasma was separated from collected blood. Three mL of distilled water was added to Reagent (1) i.e., phosphate buffer, pH 7.4, 52 mmol/L and 5-5'-Dithiobis-2-nitrobenzoic Acid. 1.5 mL of distilled water was added to Reagent (2) i.e., S-Butyrylthiocholie iodide, 218 mmol/L. In 10 µL plasma sample 1 µL and 30 µL of reconstituted reagent (1) and reagent (2) respectively were added, solution was mixed immediately. This reaction liberates 5- thio-2-nitrobenzoic acid with the formation of a strong yellow color. Rate of change of absorbance in presence and absence of test drugs was done using Auto analyzer at 405 nm. Drug solutions of required strength were prepared in distilled water. Drugs used for the study included benazepril (8.72×10^{-6} M) and physostigmine salicylate (5×10^{-6}).

In vitro *Transcorneal Permeation of Benazepril*

An all glass diffusion cell consisting of a receptor cell and donor cell was used for permeation studies. The apparatus was a modified version of the Franz diffusion cell used by Fu and Lidgate [21, 22]. The receptor cell had an internal volume of 10 mL and side arm allowed sampling of receptor fluid. The donor cell was clamped onto the top of the receptor cell. Water at 37ºC was circulated through the water jacket surrounding the receptor cell and Teflon coated magnetic stir bar kept at the bottom of the receptor cell created a homogeneous receptor volume. Whole eye balls of goat were transported from the butcher's shop to laboratory in cold (40ºC) normal saline. The cornea was carefully removed along with 2-4 mm of surrounding scleral tissue and washed with cold saline. The corneas were kept in cold freshly prepared solution of phosphate buffer (pH 7.4). Fresh cornea was mounted by sandwiching the surrounding scleral tissue between clamped donor and receptor cell. The receptor was filled with phosphate buffer (pH 7.4) solution. One mL 0.1% benazepril solution in normal saline was placed on the cornea in donor cell, while the receptor fluid was kept under stirring and permeation was continued for 120 min. Samples (2 mL) each were withdrawn from receptor cell at 5, 10, 15, 30, 45 and 60 min, replacing withdrawn sample with fresh buffer solution. Samples were analyzed by measuring an absorbance at 242.2 nm in a spectrophotometer (Perkin-Elmer EZ 301).

Biochemical Estimation of ACE in Aqueous Humor

Benazepril (0.1%) was instilled into the left eye of the rabbits while right eye served as control. One hour after instillation of the drug, aqueous humor was withdrawn from the anterior chamber of the both eyes, under ketamine anaesthesia (50 mg/kg i.v.), using 30 gauge needle and a tuberculin syringe. The samples were collected in Eppendorf's tube and immediately stored at -70ºC until analysis. The method used by Lieberman [23] was used for the determination of ACE activity in the

Table 1. Corneal permeation study of benazepril.

S.No	Time in Min.	Amount of Drug in mcg (n=6)
1	5	23.16 ± 1.06
2	10	26.39 ± 0.90
3	15	26.32 ± 1.47
4	30	17.36 ± 0.60
5	45	12.55 ± 1.03
6	60	12.11 ± 0.85

aqueous humor. Briefly the method can be described as follows: 0.15 mL of sample, when treated with hippuryl-L-histidyl-L-leucine (12.5 mM in potassium phosphate- sodium chloride buffer, pH 8.3) produced hippuric acid. Hippuric acid was then extracted with ethyl acetate and then dissolved in 3 mL of 1 M sodium chloride. The absorbance of this solution was then read after 15 min at 228nm on spectrophotometer (Perkin-Elmer EZ 301), set to zero against distilled water.

Statistical Analysis

All results were analyzed statistically using student's t-test and the value of p less than 5% ($p < 0.05$) was considered statistically significant.

RESULTS

The instillation of benazepril (0.1%) into the normotensive rabbit eyes produced a significant fall in normal IOP as compared to control (Fig 1). Infusion of 5% glucose solution produced a transient elevation of IOP up to 40-45 mmHg Prior treatment with benazepril (0.1%), in left eye, prevented such a rise significantly (Fig 2). Injection of alpha-chymotrypsin to the posterior chamber of rabbit eye produced a stable rise in IOP. Topical administration of benazepril (0.1%) produced a significant fall in IOP with peak effect achieved in first hour and effect lasting for more than 4 hrs (Fig 3).

Effects on Cholinergic Mechanisms and ACE Activity

Acetylcholine produced dose-dependent contractions on rat ileum, Benazepril (8.72×10^{-6} M) significantly potentiated the effect of acetylcholine (Fig 4). Physostigmine produced an inhibition of the enzyme cholinesterase (% inhibition from control -44.96 ± 2.54). Like physostigmine, benazepril also produced a significant inhibition of the enzyme (% inhibition from control – 23.16 ± 1.62). Benazepril produced inhibition of the enzyme cholinesterase only at high doses. Significant amount of ACE activity (6.55 ± 0.25 Units/mL) was detected in aqueous humor and serum of rabbits. Topical administration of benazepril (0.1%) produced significant decrease (4.57 ± 0.42 Units/mL; $p < 0.01$; n=6) in ACE activity in aqueous humor.

In vitro *Transcorneal Permeation*

Benazepril permeated through cornea from 0.1% solution, after 15 min permeation was maximum, than there was a decrease in permeation profile (Table 1).

DISCUSSION

Results of the present investigation demonstrate the effectiveness of benazepril in glaucoma. The effect of benazepril on normotensive rabbit eye was brief and lasted for about one and half hour only. However, the ocular hypertensive effect lasted for more than 4 hrs. in alpha-chymotrypsin induced chronic model of glaucoma in rabbits. The magnitude of lowering of IOP was also higher in this model. The normal compensatory mechanisms might be responsible for shorter duration of effect in normotensive rabbit eyes. Though with the modification of several factors like pH and solubility enhancers, corneal permeation can be improved [22], which account for its oculohypotensive action.

ACE inhibitors cause reduction in the production of aqueous humor possibly by reducing the blood flow to ciliary body [23]. ACE activity has been demonstrated in aqueous humor and other ocular tissues by various authors [10, 11, 24] Wagner et al., [12] reported their findings in enucleated human eyes, using the molecular biology technique of reverse transcription, polymerase chain reaction for expression of all components of the renin angiotensin system in individual eye samples and a RNAase protection assay to detect renin-mRNA in pooled tissue samples. They demonstrated gene expression of renin, angiotensinogen and ACE in various parts of the human eye. Although physiological significance is not known, the ACE levels are reported to be increased in sarcoid glaucomatous inflammation [21]. Lotti and Pawlowslki [6] suggested that benazepril by its ability to prevent breakdown of bradykinin promoted synthesis of prostaglandins, which in turn, could lower IOP by increasing the uveoscleral outflow without affecting the conventional trabecular meshwork outflow [8,9]. This suggests that benazepril can lower IOP even when it is not elevated. In the present investigation we found that benazepril produces a significant reduction in IOP in normotensive rabbits suggesting ACE inhibitors to be useful in low pressure glaucoma also. ACE inhibitors have been reported to affect the autonomic nervous system at various levels. ACE inhibitors lower blood pressure without causing reflex tachycardia [24]. There has been suggestions that absence of reflex tachycardia is related to enhanced cardiac parasympathetic activity, which may be due to an action of the converting enzyme inhibitors within the central nervous system or on peripheral cholinergic transmission [25, 26, 27]. Moursi et al [28] suggested converting enzyme inhibitors cause sensitization of baroreceptor reflex, possibly, by inhibiting the formation of angiotensin-II in the brain.

A facilitatory effect of antiangiotensin drugs on vagal bradycardia in pithed rats and guinea-pig has also been reported [16]. In the present study benazepril was found to potentiate the acetylcholine induced contractile responses in rat ileum. Significant inhibition of cholinesterase enzyme was observed on biochemical estimation. This suggests that benazepril possesses significant anticholinesterase activity which may be at least partly responsible for the absence of reflex tachycardia while lowering blood pressure. Also, the ocular hypotensive

activity found with benazepril may be partly because of cholinesterase enzyme inhibition. Physostigmine, a well established antiglaucoma agent acts through similar mechanism but its effects are associated with miosis. No such miosis was observed in our study. The involvement of ACE inhibiting property or increased synthesis of prostaglandins or some other mechanisms may be responsible for the absence of miosis with benazepril. Further studies to elucidate exact mechanism of this action are necessary. Transcorneal permeation study suggests that benazepril has good lipophilic properties and can cross cornea in substantial amount. In conclusion our data suggest that benazepril produced significant lowering of IOP in normotensive as well as hypertensive rabbit eyes. Inhibition of ocular ACE (Kininase II) activity could be one of the mechanisms for its action. In addition cholinesterase inhibition by benazepril may be involved in its IOP lowering effects.

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