Ocular hypotensive effects of topically administered agmatine in a chronic ocular hypertensive rat model

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Abstract

Agmatine, a primary polyamine and potential neuromodulator, exhibits a high affinity to the \( \alpha_2 \)-adrenergic receptor as well as imidazoline receptors. As \( \alpha_2 \)-adrenergic receptor agonists display positive ocular hypotensive effects, we assessed whether agmatine effectively lowers intraocular pressure (IOP) using a chronic ocular hypertensive rat model. We raised IOP in unilateral eyes of Sprague–Dawley rats by cauterizing three episcleral veins per eye. Four weeks later, we topically administered \( 10^{-3} \) M agmatine solution 4 times a day for 6 consecutive weeks. After confirming the recovery of IOP to pretreatment level at 13 weeks after cauterization, the retinal ganglion cells (RGCs) were retrogradely labeled and counted. Eyes subjected to episcleral vein cauterization (EVC) demonstrated significant increases in IOP (48.39% increase over baseline IOP), and the elevated IOP was well maintained until 12 weeks. Topically administered agmatine powerfully lowered IOP to 30.29% of its pretreatment level, and the associated washout period was about two weeks. EVC was associated with a 55.44% loss of RGCs in the control group, but agmatine appeared to attenuate this RGC loss to 18.65%. Overall, topically administered agmatine appeared to effectively lower IOP and rescue RGCs in a chronic ocular hypertensive rat model. Although the mechanism underlying these effects is not yet established, it is possible that agmatine offers a powerful new ocular hypotensive agent for eyes with chronic ocular hypertension and/or glaucoma.

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1. Introduction

Glaucoma, the leading cause of permanent blindness worldwide (Resnikoff et al., 2004; Quigley and Broman, 2006), is a progressive optic neuropathy characterized by loss of retinal ganglion cells (RGCs) and their axons (Caprioli, 1989; Nickells, 1999; Quigley, 1999). As elevated intraocular pressure (IOP) is known to be a major risk factor for glaucoma development and subsequent visual field deterioration (Mao et al., 1991; The AGIS Investigators, 2000; Heijl et al., 2002), the primary clinical concern for treatment of glaucoma is lowering IOP.

Since 1977, when timolol maleate, a nonselective \( \beta \)-adrenergic receptor blocker, was first introduced (Zimmerman and Kaufman, 1977), the topical ocular hypotensive agents have become mainstream components of glaucoma management (Strutton and Walt, 2004; van der Valk et al., 2005). In the past two decades, various eyedrop treatments including brimonidine tartrate, a selective \( \alpha_2 \)-adrenergic agonist (Serle et al., 1991), dorzolamide, a carbonic anhydrase inhibitor (Wang et al., 1990), and latanoprost, a prostaglandin analog (Camras et al., 1992), were developed and became widely used. Specifically, \( \alpha_2 \)-adrenergic receptor agonists have been reported to have ocular hypotensive and possible neuroprotective effects (Yoles et al., 1999; Ahmed et al., 2001; Wheeler et al., 2001; Gao et al., 2002).

Agmatine is an endogenous polyamine and putative neuromodulator, which exhibits affinities to imidazoline receptors. Since agmatine also acts as an \( \alpha_2 \)-adrenergic receptor agonist, N-methyl-D-aspartate inhibitor (NMDA) receptor antagonist, and neuronal/inducible nitric oxide synthase (NOS) inhibitor (Reis and Regunathan, 2000; Halaris and Plietz, 2007), it may protect RGCs from glaucomatous injuries via ocular hypotensive and neuroprotective pathways (Hartwick, 2001; Wheeler et al., 2001; Kalapesi et al., 2005; García-Campos et al., 2007). Agmatine is expected to be a powerful anti-glaucoma agent, and researchers have conducted several studies and presented in vitro data providing evidence for the neuroprotective effects of agmatine (Hong et al., 2007, 2008, 2009; Iizuka et al., 2008), but in vivo data for the ocular hypotensive effects of agmatine are lacking.
The aim of this study was to investigate the ocular hypotensive effects of topically administered agmatine in a chronic ocular hypertensive rat model.

2. Materials and methods

2.1. Animals

A total of 56 male Sprague–Dawley rats (6 weeks old, 150–170 g) were used and euthanized at the end of the study: 16 animals were sacrificed for a baseline corneal safety study of topically applied agmatine, 10 animals were used to set up the chronic ocular hypertension model, and 30 animals were used for the agmatine treatment study. Animals were maintained in a 12:12 light–dark cycle with standard food and water provided ad libitum. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Institutional Animal Care Committee. All efforts were made to minimize the number of animals sacrificed and their suffering.

2.2. Optimal concentration of topically administered agmatine

To evaluate the corneal safety of topically applied agmatine and to determine the optimal concentration of agmatine for treatment, agmatine (Sigma, St. Louis, MO)–containing eyedrops were formulated at concentrations of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$ M in preservative-free 0.1% hyaluronic acid (HA) ophthalmic solution (Santen Pharmaceutical Co., Ltd., Osaka, Japan). Each concentration of agmatine eyedrops was applied to the right eye of two rats, and the 0.1% HA vehicle was applied to their left eye, four times daily for seven consecutive days.

Animals were anesthetized with intraperitoneal injections of ketamine and xylazine (Cornell Center for Animal Resources and Education, 2008), their corneas examined with a slit-lamp biomicroscope, and IOP measured (see next section) before and after the administration of agmatine. Finally, the animals were sacrificed and all eyeballs were collected by enucleation for histological and morphological analysis (Zagon et al., 2006). The eyeballs were fixed in 4% paraformaldehyde solution for 24 h and prepared for paraffin-embedding. Sections (4-μm thickness) were stained with hematoxylin and eosin (H&E) and the histological characteristics were examined by light microscopy (Zagon et al., 2006; Yu et al., 2006).

We determined the optimal concentration of topically administered agmatine by identifying which concentration of agmatine yielded sufficient ocular hypotensive effects (IOP consistently lowered by more than 20% of baseline IOP) without causing corneal toxicity in normal rat eyes. Thus, we decided to use $10^{-3}$ M agmatine for subsequent experiments.

2.3. Intraocular pressure monitoring

As Sprague–Dawley rats are generally docile, after a short period of training, we were able to measure IOPs without sedation under topical anesthesia only (0.5% proparacaine ophthalmic solution, Alcon Laboratories, Inc., Fort Worth, TX) using a hand-held digital tonometer (Tono-Pen XL Applanation Tonometer, Reichert Inc., Depew, NY) (Urcola et al., 2006; Nissiros et al., 2008). To measure IOP, the probe tip was repeatedly touched lightly and briefly perpendicular to the central cornea. For each eye, we recorded average IOP values for several acceptable consecutive measurements, with a coefficient of variation less than 5%, four times and averaged them. All measurements were taken at the same time of day to avoid circadian IOP changes. We checked the IOP of both eyes before and 1 h after cauterization and weekly afterwards.

2.4. Rat model for chronic ocular hypertension

Chronic ocular hypertension was induced by episcleral vein cautereziation (EVC). After the animals were deeply anesthetized with ketamine and xylazine, an incision was made through the conjunctiva and Tenon’s capsule on the limbal periphery. The episcleral veins in the right eye were identified by their location in relation to the extracocular muscles; three of them (two dorsal veins and one temporal ventral vein) were cauterized with a hand-held ophthalmic cautery (Electric Eye Cautery, Rumex International Co., St. Petersburg, FL) under a surgical microscope (Shareef et al., 1995; Mittag et al., 2000). The left eye received sham surgery (only conjunctival incisions without cautereziation). After the surgical procedure, a drop of antibiotic eyedrops (0.5% levofloxacin, Santen Pharmaceutical Co., Ltd.) was topically applied. Great care was taken to minimize blood loss and avoid damage to the conjunctiva and the underlying sclera. Only those eyes that did not display scleral burns with subsequent necrosis or any complications from surgery when observed one week later were considered. Successful EVC was defined as (1) IOP greater than 50 mmHg at 1 h post-cauterization, and (2) IOP elevation greater than 30% of baseline IOP and no cautereziation-associated complications one week after cautereziation.

2.5. Topical agmatine administration for chronic ocular hypertension

Four weeks after EVC, $10^{-3}$ M agmatine eyedrops were administered to the right eye of the agmatine group four times daily for six consecutive weeks, and the 0.1% HA ophthalmic solution was administered to the control group. In both groups, 0.1% HA ophthalmic solution was instilled to the left eye at the same time points.

Then, to evaluate the washout period of topically applied agmatine and to verify the return of IOP to the pretreatment level, the IOP was monitored for another three weeks. Because the ocular hypertensive effect of EVC seemed to decrease after 12 weeks, we tried to finish all experiments before that time.

2.6. Retrograde labeling and quantification of retinal ganglion cells

RGCs were labeled by retrograde transport using crystals of 3000 MW dextran tetramethylrhodamine (DTMR, Molecular Probes, Inc., Eugene, OR) (Fritsch, 1993; Hare et al., 2001; Wolde-Mussie et al., 2001; Bakalash et al., 2007; Sposato et al., 2008), a hydrophilic neuronal tracer dye. Six eyes for each study group (EVC only, agmatine treatment after EVC, no treatment control) were included to this retrograde labeling procedure. Briefly, the animals were deeply anesthetized and the optic nerve was exposed. A longitudinal incision was made on the optic nerve sheath with a 20-gauge microvitreoretinal blade, and the optic nerve was completely transected at least 3 mm behind the globe. The transection was carefully performed to avoid traumatizing the blood vessels that supply the optic nerve. Crystals of DTMR were then directly applied into the intraorbital portion of the optic nerve.

Twenty-four hours after the application of DTMR, the eyeballs were removed by enucleation along with the optic nerves and fixed with 4% paraformaldehyde solution for 1 h. Retinas were detached from the eyes, flattened with four radial cuts (at superior, inferior, temporal and nasal poles), and mounted vitreal side up on glass slides. A total of 12 fields for each retina (peripapillary, middle, and peripheral fields per retinal quadrant) were subjected (Yu et al., 2006) and assessed by fluorescence microscopy under 200x magnification by an observer who was blinded to the identity of the retinas. The number of labeled cells was divided by the area of the
region and pooled to calculate the mean density of labeled RGCs for each retina.

2.7. Statistical analysis

Data are expressed as means ± S.D. for the IOP recordings and the RGC densities. Data were compared between groups with the Mann–Whitney U test using the SPSS program for Windows, version 12.0.1 (SPSS Inc., Chicago, IL). P-values < 0.05 were considered statistically significant.

3. Results

3.1. Optimal concentration of topically administered agmatine

Initially, the rats (n = 16) were examined with a slit-lamp bimicroscope and their IOPs were checked under general anesthesia. All of them were comparable in ocular surface morphology and had good corneal reflex. Mean IOP was 9.88 ± 2.55 mmHg (range, 6–16) for right eyes and 9.81 ± 2.54 mmHg (range, 6–15) for left eyes (p = 0.985).

After seven-day applications of agmatine, the rats' eyes were reevaluated. Agmatine concentrations of 10⁻¹ M caused rat corneas to become hazy and lose corneal reflex. However, corneas retained their normal appearance when treated with lower agmatine concentrations (10⁻²–10⁻⁸ M). In histological and morphological analysis, only the 10⁻¹ M agmatine application caused toxic changes to corneas (Fig. 1) including thinning and destruction of stratified epithelium, less organized matrix in the stroma, and abnormal cellular proliferation and neovascularization on the endothelial layer. In contrast, lower concentrations of agmatine of 10⁻²–10⁻⁸ M did not cause any significant changes to the central and peripheral cornea. The 10⁻³ M or higher concentrations of agmatine consistently lowered IOP by more than 20% of the baseline IOP (Fig. 2). Therefore, we selected the 10⁻³ M concentration of agmatine, which showed no corneal toxicity and sufficient ocular hypotensive effects on normal rat eyes, for subsequent experiments.

3.2. Chronic ocular hypertension rat model

Before verifying the effects of agmatine, the success rate and durability of EVC induced chronic ocular hypertension rat model were confirmed. Using the success criteria described above in Section 2.4, the overall success rate of EVC was 65%. In detail, the IOP of all 40 eyes peaked at higher than 50 mmHg 1 h after cauterization, but 14 eyes (35%) fell into phthisis one week later.

IOP changes after successful EVC are depicted in Fig. 3. One week after EVC, IOP was elevated from 15.50 ± 1.73 mmHg to 23.00 ± 2.16 mmHg (48.39% increase of baseline IOP) and lasted for 12 weeks (IOP, 28.00 ± 3.16 mmHg). After 12 weeks, however, IOP gradually decreased. Since the elevated IOP caused by EVC was maintained for only 12 weeks, we scheduled all further studies evaluating the ocular hypotensive effects of agmatine to finish before 12 weeks.

3.3. Ocular hypotensive effects of topically administered agmatine

To confirm the success of EVC, the application of agmatine and 0.1% HA vehicle was started four weeks after cauterization (Fig. 4).

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Fig. 1. Corneal safety of topically applied agmatine. (A) Control eyes, (B) 10⁻¹ M (C) 10⁻² M, and (D) 10⁻⁴ M agmatine. Total magnification was 200×. Asterisk refers to neovascularization on endothelial side of cornea.
In the agmatine group, the IOP was 28.79 ± 4.02 mmHg before medication, fell to 21.86 ± 2.28 mmHg (24.07% decrease) after two weeks of application, and to 20.07 ± 2.09 mmHg (30.29% decrease) after six weeks of application. In the control group, the IOP did not significantly change with time.

3.4. Protective effects of agmatine on retinal ganglion cells

Representative retrograde RGC labeling is shown in Fig. 5. A total of 12 fields for each retina (peripapillary, middle, and peripheral fields per retinal quadrant) were assessed and the mean density of labeled RGC was calculated for each retina. In control eyes, it was 18.38 ± 2.42 cells/1000 µm². By EVC, it decreased to 8.19 ± 2.18 cells/1000 µm² (55.44% loss of control eyes; \( p < 0.001 \)). Topical agmatine treatment attenuated this RGC loss to 14.95 ± 2.94 cells/1000 µm² (18.65% loss of control eyes; \( p = 0.001 \)).

4. Discussion

In the present study, topically applied agmatine showed powerful ocular hypotensive effects in a rat model of chronic ocular hypertension induced by cauterization of three episcleral veins. This model was first introduced by Shareef et al. (1995) and has been widely used in many in vivo glaucoma studies. EVC effectively raises IOP, but does not cause any other adverse changes in the anterior chamber or ciliary body (Nissirios et al., 2008). However, the duration of successful EVC has been variously reported to last anywhere from two to six months (Sawada and Neufeld, 1999; Kanamori et al., 2005; Urcola et al., 2006; Vidal et al., 2006). Diversity of the strain, age, and gender of rats, the number of cauterized episcleral blood vessel trunks, other details of the cauterization method, and postcauterization medication may explain this variation. In our study, the overall success rate of EVC was just over 60%, and even in successful eyes, elevated IOP lasted for just 12 weeks. Our model was comparable with those of previous reports (Sawada and Neufeld, 1999; Kanamori et al., 2005; Urcola et al., 2006; Vidal et al., 2006).

When episcleral veins are cauterized, the outflow facility of aqueous humor is obstructed and IOP is elevated. Even though our overall success rate was about 60%, successful eyes identified one week after cauterization showed stable progress thereafter. Because IOP peaked above 50 mmHg immediately after cauterization and remained high during the early days postcauterization (Shareef et al., 1995), the function of the ciliary body may have shut down, causing more susceptible eyes to fall into phthisis. It is possible that eyes capable of withstanding such adversity successfully maintain high IOP for a long time.

We measured IOP under general anesthesia for the baseline safety study, but under topical anesthesia for further chronic ocular hypertension studies. Baseline IOPs differed by anesthesia method, with means of 9.88 ± 2.55 mmHg and 15.50 ± 1.73 mmHg for general and topical anesthesia, respectively. This difference seems to originate in the effects of anesthetic agents and/or stress. In any case, these differences are acceptable as a consistent method was used for each model.

Agmatine, an endogenous polyamine that is metabolized from L-arginine by decarboxylation, has various biological functions (Sener et al., 1989; Li et al., 1994; Kalra et al., 1995; Galea et al., 1996; Kolesnikov et al., 1996; Dukdakowska et al., 2003; Su et al., 2003; Grillo and Colombatto, 2004). Recently, the neuroprotective effects of agmatine on CNS injuries have attracted attention of many neuroscientists (Gilad et al., 1996; Olmos et al., 1999; Gilad and Gilad, 2000; Yu et al., 2000; Zhu et al., 2003; Kim et al., 2004; Kotil et al., 2006; Wang et al., 2006). Researchers have reported agmatine's
protective effects against oxidative injuries measured via observation of RGCs in vitro (Hong et al., 2007, 2008, 2009; Iizuka et al., 2008). We evaluated the ocular hypotensive effects of agmatine. Agmatine has a good affinity to the $\alpha_2$-adrenergic receptor and plays a role as an agonist, so we suggested that it may have IOP lowering effects like brimonidine. We found that administration of agmatine lowered IOP and attenuated the loss of RGCs in our chronic ocular hypertensive rat model. We also observed that topically applied agmatine attenuated the pressure-induced cytotoxicity of RGCs via retrograde RGC labeling, although the mechanism of this protection on RGCs is not yet clear. It may simply result from pressure lowering effect or direct neuroprotection of agmatine. Further experiments are needed to confirm whether agmatine also directly protects RGCs in an in vivo glaucoma model.

High concentrations of agmatine ($10^{-3} \text{ M}$) caused apparent corneal toxicity that appeared to be due to inflammation. We observed thinning and destruction of stratified epithelium, less organized matrix in the stroma, and abnormal cellular proliferation and neovascularization on the endothelial layer. Further studies are needed to understand these toxic mechanisms. We formulated agmatine-containing eyedrops using preservative-free 0.1% HA ophthalmic solution rather than normal saline or distilled water because the HA-based solution resulted in better corneal reflex in our baseline study (data not shown).

![Fig. 4. Ocular hypotensive effects of topical agmatine on chronic ocular hypertension rat model. AG = agmatine; EVC = episcleral cauterization; HA = hyaluronic acid; IOP = intraocular pressure; LE = left eye; RE = right eye. *Topical agmatine application period.](image)

![Fig. 5. Retinal ganglion cell protective effects of topical agmatine on chronic ocular hypertension rat model. (A) Control eyes, (B) eyes received episcleral vein cauterization only, and (C) eyes received episcleral vein cauterization and topical agmatine treatment. Total magnification was 200×.](image)
In conclusion, topically administered agmatine effectively lowered IOP and rescued RGCs in a chronic ocular hypertensive rat model. Although we were unable to identify the underlying mechanisms, agmatine may work as a powerful new ocular hypertensive agent in eyes with chronic ocular hypertension and/or glaucoma.

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References


