Lycium Barbarum Polysaccharides Rescue Neurodegeneration in an Acute Ocular Hypertension Rat Model Under Pre- and Posttreatment Conditions

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PURPOSE. To investigate the posttreatment neuronal rescue effects of Lycium barbarum polysaccharides (LBP) in an acute ocular hypertensive (AOH) model.

METHODS. Intraocular pressure (IOP) was elevated manometrically to 80 mm Hg (AOH) or 15 mm Hg (sham) for 120 minutes in adult Sprague-Dawley rats. Five experimental groups were considered: Three AOH groups were pretreated with PBS (vehicle) (n = 9), LBP 1 mg/kg (n = 8), or 10 mg/kg (n = 13), and one AOH group was posttreated with LBP 10 mg/kg (n = 8), once daily. The sham cannulation group (n = 5) received no treatment. Pretreatments commenced 7 days before and posttreatment 6 hours after AOH, and continued up through postcannulation day 28. All the animals underwent optical coherence tomography and electroretinogram measurements at baseline and postcannulation days 10 and 28. The ganglion cell layer (GCL) densities were quantified at day 28.

RESULTS. Both inner retinal layer thickness (IRLT) and positive scotopic threshold response (pSTR) underwent significant reduction (~50% of thickness and amplitude) in the vehicle group (P < 0.05). Pretreatment with LBP 1 and 10 mg/kg retained 77 ± 11% and 89 ± 8% of baseline IRLT, respectively, and preserved pSTR functions. The posttreatment group showed a significant reduction in IRLT (~35 ± 8%, P < 0.001) and pSTR (~48% of baseline, P < 0.001) on day 10. By day 28, there was an improvement in functional pSTR (~72% of baseline, P > 0.05) with no significant further thinning (~40 ± 8%, P = 0.15) relative to day 10. GCL density was reduced in vehicle control (P = 0.0001), but did not differ between sham and pre- and posttreated AOH groups.

CONCLUSIONS. The rescue effect of LBP posttreatment was observed later, which arrested the secondary degeneration and improved the retinal function.

Keywords: lycium barbarum polysaccharides, neuronal rescue, posttreatment, acute ocular hypertension, rat model

Neuroprotection is considered as a potential treatment option for glaucoma due to the progressive nature of the disease with multifactorial contributions and because of the similarities it shares with other central nervous system disorders.1,2 In recent years, several possible neuroprotective agents have been identified for glaucoma treatment, which target factors other than the elevated intraocular pressure (IOP).3,4 However, only a few of these agents underwent clinical trials and none have so far been approved for clinical use.

The fruits of Lycium barbarum, also known as wolfberry, contain many bioactive substances including polysaccharides, carotenoids, flavonoids, amino acids, vitamins, fatty acids, and other trace elements.3 Interestingly, the polysaccharides of L. barbarum (LBP) have been shown to have positive effects on aging processes,5,6 fatigue,6 oxidative stress,7–12 neurodegenerative disease,13,14 tumor growth,15 immune response,16 inflammation,17 irradiation,18 hyperglycemia,19 hyperlipidemia20 and other induced toxicities.17,21 The neuroprotective effects of LBP have been demonstrated in different experimental models of central nervous system disorders that include variants of optic neuropathies,22–29 The possible neuroprotective mechanisms of LBP in preserving the retinal ganglionic cells (RGC) have been investigated in rodent models of chronic ocular hypertension,22–24 acute ocular hypertension (AOH),26,29 partial optic nerve transection,25,27 and ischemic reperfusion injuries.28 However, all these studies demonstrated the prophylactic effect of LBP by pretreating the animals from 1 week prior to the insult until the end of the study period. Only one of these studies applied functional testing,29 the rest adopting structural end points, which were assessed using histologic techniques. While longitudinal data were collected by cross-sectional observation of animals killed at different time points, there have been no reports demonstrating the neuroprotective effect of LBP using in vivo longitudinal structural evaluation or combined structure–function tools.
Recently, there is an increasing trend of adopting bed- to bench-side techniques in preclinical research for longitudinal structural and functional assessment. Optical coherence tomography (OCT) has been reliably applied in small animals to study and longitudinally quantify retinal structure. Also, variations of electroretinogram (ERG) protocols have been tested to differentially measure the inner and outer retinal response in rodent experimental models. Importantly, the posttreatment efficacy of any neuroprotective drug should be explored at the level of preclinical research to simulate the conditions of clinical research in which the drug would be trialed in an established disease condition with varying severity. Consideration of studies using LBP revealed that its protective effects in animal models of optic neuropathies. In view of these limitations, an AOH rat model was considered in this study to investigate the longitudinal neuroprotective efficacy of LBP using combined structural OCT and functional ERG measurements under pre- and posttreatment conditions.

METHODS

Animals
Adult female Sprague-Dawley (SD) rats were housed at room temperature (20°C) under normal lighting conditions (approximately 200 lux) with an alternating light/dark cycle (12 hours light/12 hours dark). Both food (PicoLab diet 20 (5053); PMI Nutrition International, Richmond, IN, USA) and water were supplied ad libitum. All experimental procedures and care involving the animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Animal Ethics Sub-committee of The Hong Kong Polytechnic University.

Experimental Design
Sixty 10-week-old rats (180–200 g) were randomly allocated into five experimental groups: (A) AOH vehicle control, pretreatment with phosphate-buffered saline (PBS); (B) AOH pretreatment with LBP 1 mg/kg; (C) AOH pretreatment with LBP 10 mg/kg; (D) AOH posttreatment with LBP 10 mg/kg; and (E) sham control (Fig. 1). For pretreatment groups, animals were fed with LBP or PBS once daily, from 7 days prior to the AOH insult until the end of the study period at postcannulation day 28. For the posttreatment group, LBP feeding was initiated 6 hours after the AOH insult and continued until the end of the study period at day 28. All experimental groups underwent structural OCT and functional ERG measurements at baseline and postcannulation day 10 and day 28. Rats were killed at day 28 by inhalation of CO2 and retinal sections were collected for morphologic examination.

Induction of Acute Ocular Hypertensions
Animals were anesthetized with a mixture of 60 mg/kg ketamine 10% (Alfasan International B.V., Woerden, Holland) and 5 mg/kg xylazine 2% (Alfasan International B.V.) via intraperitoneal injection. The anesthesia was maintained for 120 minutes by injecting half the initial dosage every 45 minutes via the same route. The animal's body temperature under anesthesia was maintained using a heating mat. Following the application of a drop of topical anesthetic (Provain-POS 0.5% wt/vol eye drops; URSAHARM, Saarbrücken, Germany) and dilatation drops (Mydriacyl 1% eye drops; Alcon-Couvreur, Puurs, Belgium), the anterior chamber of one eye was cannulated using a 30-gauge needle attached to a tubing connected to a reservoir containing Gibco Hank's balanced salt solution (HBSS; Thermo-Fisher Scientific, Waltham, MA, USA) via a pressure transducer (60-5005; Harvard Apparatus, Holliston, MA, USA). The target IOP of either 15 mm Hg for the sham control group or 80 mm Hg for AOH groups for 120 minutes was achieved manometrically by adjusting the height of the reservoir (Fig. 2 shows representative pictures of anterior chamber cannulation and fundus photographs). The IOP was also monitored intermittently using the Tonolab Tonometer TV02 (Icare, Vantaa, Finland). To prevent corneal dehydration, Lacryvisc gel (Alcon, Rueil-Malmaison, France) was applied throughout the experimental period. Following the 120-minute AOH period, the pressure in the reservoir was gradually lowered to the IOP of 15 mm Hg and the needle was gently removed. The eyes were then treated with topical antibiotic eye drops (gentamycin; Gibco, Thermo-Fisher Scientific). Approximately 15% of the animals developed complications, including lens puncture, corneal haze, and vitreous hemorrhage; these were excluded from the study.
Anterior chamber of rats were cannulated with a 30-gauge needle (A, B), and fundus photographs (C, D) were captured immediately after sham (A, C) or AOH cannulation (B, D). When compared with sham, AOH cannulation showed distension of anterior chamber (due to fluid inflow), changes in iris color from pink to pale (arrows), and compromised retinal blood flow (D, pale appearance of fundus) and thinning of blood vessels.

**Drug Administration**

The process of extracting polysaccharides from Lycium fruits has been described in detail elsewhere.35 The LBP (the polysaccharides of *Lycium barbarum*) solution was freshly prepared by dissolving the dried LBP powder in PBS. Animals that underwent sham cannulation received no feeding, whereas animals in the AOH cannulation groups were pre- or posttreated with PBS, 1 mg/kg LBP, or 10 mg/kg LBP solution once daily (between 10:30 and 11:30 AM) using a nasogastric feeding tube. The total treatment periods for pre- and posttreatments were 35 and 28 days, respectively.

**Electroretinography**

Electoretinal responses were measured using a full-field Ganzfeld (Q450; RETI Animal, Roland Consult, Brandenburg an der Havel, Germany). Animals were dark adapted overnight (>12 hours) prior to the ERG measurement. Preparation for ERG was done under dim red light to maintain the dark-adapted state of the animals. Following a similar anesthetic and dilation regimen as for AOH induction, the animal was placed on a platform that was connected to a warm water bath to maintain the body temperature at around 37°C during the recording period. A drop of lubricating gel was applied on the corneal surface to prevent dehydration. The electrode placements for ERG recording were as follows: a pair of gold ring electrodes of 4 mm diameter (Roland Consult) placed on the corneal surface of both eyes serving as active electrodes; needle electrodes (Item No. U51-426; GVB-geliMED, Bad Segeberg, Germany) inserted into the lateral canthi of each eye to serve as references; needle electrode inserted into the base of the tail to serve as a ground electrode. An impedance of less than 10 KΩ active electrodes was maintained during the recording period.

White light-emitting diode (LED) light was chosen as source for flash stimuli. ERG responses were recorded for a subset of animals ($n = 10$) using flash intensities ranging from $-5.7$ to $1.5 \log U$ ($\log U = \log \text{cd.s.m}^{-2}$). The signals were recorded with a bandpass filter ranging from $0.1$ to $1000 \text{ Hz}$. Results from the subset of animals (Supplementary Fig. S1) showed that the positive scotopic threshold response (pSTR) was observed around $-4.2 \log U$. Also, the maximum a- and b-wave responses were obtained from $1.5 \log U$. Subsequently, the experimental animals underwent STR recording from flash stimuli $-4.8$ to $-4.05 \log U$ followed by scotopic responses using $1.5 \log U$. The amplitudes and implicit time of pSTR and scotopic a- and b-wave responses were considered for the analysis.

**Optical Coherence Tomography**

Cross sections of the retina were imaged using a spectral-domain OCT (Micron IV; Phoenix Research Lab, Pleasanton, CA, USA). A circular B-scan with a radius of 0.51 mm, consisting of 1024 A-scans with axial and transverse resolution of 1.8 and 3.0 μm, was used to image the peripapillary retinal thickness (Fig. 3A). Following a similar anesthetic and dilation regimen, lubricating gel was applied on the corneal surface to improve the contact between the animal eye and objective lens. The B-scan image obtained was then analyzed using a semiautomated segmentation algorithm (Insight software, Phoenix Research Lab). The measurements of nerve fiber layer thickness (RNFLT), inner retinal layer thickness (IRLT), and outer retinal layer thickness (ORLT) were included in the analysis (Fig. 3B). IRLT was the sum of inner plexiform layer (IPL) and inner nuclear layer thickness (INL); ORLT was measured from outer plexiform layer to RPE. Mean thickness of each layer was determined by averaging data points (1024) that covered the circumference of the circle. During follow-up examinations, a circle scan was placed in close proximity to the baseline measurement by referring back to the baseline fundus image.
Retinal Histology

After OCT and ERG measurements on day 28, the animals were euthanized by CO₂ asphyxiation. The eyes were enucleated and the eye cups were collected after removing the cornea and the lens. Eye cups were fixed in 4% paraformaldehyde in PBS at room temperature overnight before paraffin embedding. Sagittal sections of 5 µm parallel to the optic nerve were collected using a microtome and stained using hematoxylin and eosin (H&E). Sections were imaged using a light microscope at ×200 magnification (Nikon, Tokyo, Japan). Approximately 250 µm away from the sceral canal opening, regions of 500 × 500 µm of central retina were selected for morphologic analysis. Cells in the retinal ganglion layer of the selected regions were counted and the corresponding retinal length was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA) for the calculation of ganglion cell layer (GCL) density/mm in retina.

Data Analysis

The data collection and quantification were performed and analyzed as blind. The results were normally distributed and are presented as means with SEM. All analysis was performed using SPSS 23.0 (IBM Corp., Armonk, NY, USA). The significance level was set at 0.05. Mixed-model ANOVA was applied to test the difference in ERG parameters (amplitudes and implicit time) and OCT measured retinal thickness between the five groups and also within the groups measured over three time points with Bonferroni post hoc correction. Also, the retinal thicknesses are presented as percentage change from baseline.

RESULTS

Optical Coherence Tomography

Figure 4 presents typical peripapillary SD OCT B-scans of a rat from each experimental group, showing the longitudinal changes in RNFL, IRLT, and ORLT from baseline to postcannulation days 10 and 28. The thickness of RNFL, IRL, and ORL of the five cohorts measured at three points (baseline, postcannulation days 10 and 28) is given in Table 1. On differential OCT thickness analysis, the IRLT showed a significant difference among the treatment groups measured over time (mixed ANOVA: interaction: P = 0.01). Figure 5A shows the temporal changes in IRLT between the treatment groups, presented as mean percentage change in thickness from baseline. While the sham control showed a transient thickening of IRL at day 10 (13.6 ± 4%, P > 0.05, relative to baseline), the AOH vehicle control showed a significant thinning on both
day 10 (−35.8 ± 11.4%, P = 0.001, relative to baseline) and day 28 (−49.7 ± 10.7%, P = 0.001, relative to day 10 and baseline).

Under pretreatment conditions, both dosages of LBP (1 mg/kg, 10 mg/kg) slowed down the rate of IRL thinning on both day 10 and day 28, but at different rates. The LBP pretreatment of 10 mg/kg showed no significant change in thickness at day 10 (−2.6 ± 7.2%, P > 0.05, relative to baseline), whereas LBP 1 mg/kg showed a trend of thinning at day 10 (−15.9 ± 11.2%, P = 0.27, relative to baseline). On day 28, the rates of IRL thinning in pretreatment with LBP 1 and 10 mg/kg were −23.0 ± 10.5% (P = 0.04, relative to day 10; P = 0.05, relative to baseline) and −11.3 ± 8.1% (P = 0.001, relative to day 10; P > 0.05, relative to baseline) as compared to vehicle control (−49.7 ± 10.7%).

Under posttreatment condition of LBP 10 mg/kg, there was a significant thinning of IRL at day 10 (−34.6 ± 7.9%, P = 0.001, relative to baseline) that was comparable to the vehicle control. However, the rate of thinning slowed down from days 10 to 28 (−39.8 ± 8.0%, P = 0.15, relative to day 10), while this was still progressive in the vehicle control group (P = 0.001). On pairwise comparison, the thickness of vehicle control was significantly reduced compared to sham control (day 10: P = 0.04; day 28: P = 0.02) and pretreatment LBP 10 mg/kg (day 28: P = 0.05); the thickness with posttreatment with LBP 10 mg/kg was significantly less than in the sham control (day 10: P = 0.02; day 28: P = 0.07).

The effects of cannulation on RNFLT and ORLT of the five experimental groups, presented as percentage change from baseline, are shown in Figures 5B and 5C. Irrespective of sham or AOH cannulation under different treatment conditions, the RNFL (Fig. 5B) showed a transient thickening (ranging from 34% to 51% from baseline) at day 10 (mixed ANOVA: time effect: P = 0.001; between groups: P = 0.07; interaction effect: P = 0.82). While the sham control showed a complete recovery to baseline thickness on day 28, there was a residual RNFL thickening present in the other four AOH cannulated groups. However, this was not significantly different from their respective baselines or between groups. The ORLT (Fig. 5C) showed a significant change within the groups measured over time and a significant difference between groups at day 10 (mixed ANOVA: time: P = 0.001; between groups: P = 0.02; interaction effect: P = 0.24). The sham control showed a small, but nonsignificant thickening of ORL on both days 10 and 28, and the rest of the AOH groups showed a significant thickening at day 10 (P = 0.001) that recovered to baseline at day 28. Significant difference in thickness between groups was noted only at day 10 (P = 0.01), with post hoc analysis showing increased thickness in vehicle control (P = 0.01) and pretreatment with LBP 10 mg (P = 0.01) as compared with sham control. However, there was no significant difference in ORLT between the pre- and posttreatment groups and also their respective baselines at day 28.

**Electroretinography.**

Table 2 summarizes the parameters in the ERG measurements, namely, pSTR, scotopic b-wave, and a-wave amplitudes obtained from the five experimental groups at baseline, postcannulation days 10 and 28. The amplitudes of pSTR showed a significant difference among groups receiving different treatments (mixed ANOVA: time: P = 0.06; between groups: P = 0.01; interaction effect: P = 0.15). The pSTR traces of representative rats are displayed in Figure 6. The pSTR responses of the sham group remained stable over the study period, but the vehicle control group showed a reduction at day 10 that dropped significantly at day 28 (P < 0.05, relative to baseline). Under pretreatment conditions, both the LBP dosages (1 and 10 mg/kg) showed a preservation of pSTR responses on both days 10 and 28, which was comparable with their respective baselines and the sham control. Under the posttreatment condition of LBP 10 mg/kg, the pSTR responses showed an initial reduction at day 10 (P < 0.05, relative to baseline), which improved subsequently at day 28, showing no difference from its baseline. On pairwise comparison, the amplitude of vehicle control was reduced significantly compared with the sham control (day 10: P = 0.02; day 28: P = 0.03) and pretreatment LBP 1 mg/kg (day 28: P = 0.05). There was no difference in functional rescue effects between the two LBP dosages (1 and 10 mg/kg) studied under pretreatment conditions.

Scotopic ERG traces from one representative rat of each experimental group measured at baseline and postcannulation days 10 and 28 are shown in Figure 6. The a-wave responses (Table 2) remained similar across groups over the course of the study period (mixed ANOVA: time: P = 0.75; between groups: P = 0.53; interaction effect: P = 0.33). The amplitudes of b-wave (Table 2) showed a significant difference among the groups (mixed ANOVA: time: P = 0.06; between groups: P = 0.001; interaction effect: P = 0.10). On pairwise comparison, the responses of the vehicle control dropped significantly as compared to sham control (day 10: P = 0.02; day 28: P = 0.04) and pretreatment LBP 10 mg/kg (day 10, P = 0.03) groups. The

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**Table 1.** Differential OCT Thickness of the Experimental Groups Measured at Baseline and Postcannulation Days 10 and 28

<table>
<thead>
<tr>
<th>Experimental groups, N</th>
<th>AOH-Vehicle Control, Pretreated, n = 9</th>
<th>AOH-LBP 1 mg/kg, Pretreated, n = 8</th>
<th>AOH-LBP 10 mg/kg, Pretreated, n = 13</th>
<th>AOH-LBP 10 mg/kg, Posttreated, n = 8</th>
<th>Sham Control, n = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRLT, mean (SEM), µm</td>
<td>73.0 (1.4)</td>
<td>72.4 (0.8)</td>
<td>71.0 (0.9)</td>
<td>73.3 (1.6)</td>
<td>71.3 (1.8)</td>
</tr>
<tr>
<td>Day 10</td>
<td>47.2 (8.8)*</td>
<td>60.9 (8.0)</td>
<td>69.1 (5.1)</td>
<td>47.2 (5.2)*</td>
<td>81.1 (4.2)</td>
</tr>
<tr>
<td>Day 28</td>
<td>37.0 (8.1)*</td>
<td>55.7 (7.5)</td>
<td>62.9 (5.7)</td>
<td>43.5 (5.3)*</td>
<td>73.0 (2.0)</td>
</tr>
<tr>
<td>RNFLT, mean (SEM), µm</td>
<td>25.3 (1.0)</td>
<td>27.2 (1.5)</td>
<td>28.4 (0.9)</td>
<td>23.9 (1.5)</td>
<td>28.3 (0.7)</td>
</tr>
<tr>
<td>Day 10</td>
<td>37.1 (2.5)*</td>
<td>40.0 (2.2)*</td>
<td>42.9 (1.6)*</td>
<td>35.4 (4.5)*</td>
<td>37.7 (4.4)*</td>
</tr>
<tr>
<td>Day 28</td>
<td>29.6 (3.5)†</td>
<td>30.4 (2.0)†</td>
<td>33.8 (2.2)†</td>
<td>26.5 (3.0)†</td>
<td>27.4 (1.1)†</td>
</tr>
<tr>
<td>ORLT, mean (SEM), µm</td>
<td>125.5 (2.0)</td>
<td>122.3 (1.8)</td>
<td>123.7 (1.1)</td>
<td>120.4 (1.6)</td>
<td>119.4 (3.3)</td>
</tr>
<tr>
<td>Day 10</td>
<td>159.4 (3.0)*†</td>
<td>131.7 (2.2)*†</td>
<td>136.8 (2.2)*‡</td>
<td>132.9 (2.4)*</td>
<td>123.1 (3.3)</td>
</tr>
<tr>
<td>Day 28</td>
<td>126.8 (4.8)*‡</td>
<td>128.6 (4.2)</td>
<td>127.4 (1.4)*†</td>
<td>121.8 (2.1)†</td>
<td>121.6 (2.4)</td>
</tr>
</tbody>
</table>

* P < 0.001 when compared to baseline.  
† P < 0.01 when compared to day 10.  
‡ P < 0.05 when compared to sham control.  
§ P < 0.05 when compared to AOH pretreatment with 10 mg/kg LBP. Bonferroni post hoc test of mixed-model ANOVA.
FIGURE 5. Mean peripapillary retinal thicknesses, namely, (A) IRLT, (B) RNFLT, and (C) ORLT were compared between the five experimental groups from baseline to postcannulation day 28. The data are presented as percentage change from baseline. Error bars: standard error of mean. *P < 0.001 versus baseline; †P < 0.01 versus day 10; ‡P < 0.05 versus sham control; §P < 0.05 versus AOH pretreatment LBP 10 mg/kg (*†‡§Bonferroni post hoc test of mixed-model ANOVA).

The present study is the first to investigate the posttreatment protective effect of LBP using combined structure–function approaches in an AOH rat model.

On longitudinal structural evaluation, AOH rats (80 mm Hg for 120 minutes) showed a progressive thinning of the inner retina with no drastic changes to the outer retinal thickness. On differential analysis of the inner retina into RNFL and IRL (INL+INL), the IRL showed a progressive thinning. The RNFL showing a transient thickening (at day 28) of RNFL and IRL (INL+INL), the IRL showed a progressive thinning.

mean GCL density (cells/mm, mean ± SEM) in AOH rats (80 mm Hg for 120 minutes) showed a progressive thinning of the inner retina with no drastic changes to the outer retinal thickness. On differential analysis of the inner retina into RNFL and IRL (INL+INL), the IRL showed a progressive thinning. The RNFL showing a transient thickening (at day 28) of RNFL and IRL (INL+INL), the IRL showed a progressive thinning. The RNFL showing a transient thickening (at day 28) of RNFL and IRL (INL+INL), the IRL showed a progressive thinning.

DISCUSSION

The present study is the first to investigate the posttreatment efficacy of LBP on a model of optic neuropathy, while other studies have shown the pretreatment outcome of LBP. In addition, the study reports the longitudinal in vivo neuroprotective effect of LBP using combined structure–function approaches in an AOH rat model.

On longitudinal structural evaluation, AOH rats (80 mm Hg for 120 minutes) showed a progressive thinning of the inner retina with no drastic changes to the outer retinal thickness. On differential analysis of the inner retina into RNFL and IRL (INL+INL), the IRL showed a progressive thinning. The RNFL showing a transient thickening (at day 28) of RNFL and IRL (INL+INL), the IRL showed a progressive thinning.

pSTR showed an insignificant trend of delayed response (P > 0.05) in vehicle control and posttreated LBP 10 mg/kg on day 28 as compared to sham control or both pretreated LBP groups (mixed ANOVA: time: P = 0.23; between groups: P = 0.06; interaction effect: P = 0.85). The b-wave implicit time showed a significant time effect (mixed ANOVA: time: P = 0.01; between groups: P = 0.81; interaction effect: P = 0.87) that was found to be delayed at day 10 as compared to baseline recordings, with no difference on day 28. The a-wave implicit time showed a significant interaction effect (P = 0.02), which on pairwise comparison showed a delay in response in posttreated LBP 10 mg/kg on day 28 (8.6 ms, P = 0.01, relative to baseline of 7.9 ms). In general, the implicit time of all ERG parameters did not show any significant changes due to the AOH or under different LBP treatment conditions.

Histology

Figure 7A shows a representative histologic cross section from each experimental group collected at postcannulation day 28. The mean GCL density (cells/mm, mean ± SEM), presented in Figure 7B, showed a significant difference between groups (P < 0.001, ANOVA). On pairwise comparison, the GCL density was significantly reduced in the vehicle control (P < 0.001) as compared to sham cannulation, pretreatment LBP 1 mg/kg, pretreatment AOH 10 mg/kg, and posttreatment 10 mg/kg AOH groups. There was no difference in GCL density between sham and pre- and post-LBP-treated AOH groups.

Table 2. Amplitudes of pSTR, Scotopic b- and a-Wave Responses of the Experimental Groups Measured at Baseline and Postcannulation Days 10 and 28

<table>
<thead>
<tr>
<th>Experimental Groups, N</th>
<th>AoH-Vehicle Control, Pretreated, n = 9</th>
<th>AoH-LBP 1 mg/kg, Pretreated, n = 8</th>
<th>AoH-LBP 10 mg/kg, Pretreated, n = 13</th>
<th>AoH-LBP 10 mg/kg, Posttreated, n = 8</th>
<th>Sham Control, n = 6</th>
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<tbody>
<tr>
<td>pSTR, mean (SEM), μV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>14.7 (2.7)</td>
<td>14.9 (2.7)</td>
<td>16.3 (2.1)</td>
<td>19.4 (2.3)</td>
<td>15.1 (2.4)</td>
</tr>
<tr>
<td>Day 10</td>
<td>6.4 (1.9)†</td>
<td>11.8 (2.6)</td>
<td>14.4 (2.7)</td>
<td>9.3 (2.4)*</td>
<td>20.3 (3.0)</td>
</tr>
<tr>
<td>Day 28</td>
<td>6.7 (1.5)**</td>
<td></td>
<td>15.4 (2.6)</td>
<td>13.8 (2.0)</td>
<td>13.9 (1.8)</td>
</tr>
<tr>
<td>b-wave, mean (SEM), μV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>580.3 (82.6)</td>
<td>559.5 (78.0)</td>
<td>632.2 (57.6)</td>
<td>640.3 (96.0)</td>
<td>704.2 (132.3)</td>
</tr>
<tr>
<td>Day 10</td>
<td>266.8 (45.8)**</td>
<td></td>
<td>541.0 (125.7)</td>
<td>719.5 (79.6)</td>
<td>726.3 (112.1)</td>
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<tr>
<td>Day 28</td>
<td>313.7 (87.9)**</td>
<td></td>
<td>548.3 (102.1)</td>
<td>540.7 (64.0)†</td>
<td>712.5 (103.6)</td>
</tr>
<tr>
<td>a-wave, mean (SEM), μV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>−257.3 (36.6)</td>
<td>−247.8 (25.4)</td>
<td>−260.0 (22.4)</td>
<td>−273.5 (40.5)</td>
<td>−280.5 (44.8)</td>
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<tr>
<td>Day 10</td>
<td>−237.3 (37.7)</td>
<td>−297.5 (40.9)</td>
<td>−354.9 (26.6)</td>
<td>−255.0 (31.1)</td>
<td>−269.1 (52.8)</td>
</tr>
<tr>
<td>Day 28</td>
<td>−212.9 (33.9)</td>
<td>−281.8 (32.4)</td>
<td>−244.5 (25.9)†</td>
<td>−276.6 (26.5)</td>
<td>−292.8 (25.5)</td>
</tr>
</tbody>
</table>

* P < 0.001 when compared to baseline.
† P < 0.01 when compared to day 10.
‡ P < 0.05 when compared to sham control.
§ P < 0.05 when compared to AOH pretreatment with 10 mg/kg LBP.
|| P < 0.05 when compared to AOH pretreatment with 1 mg/kg LBP; Bonferroni post hoc test of mixed-model ANOVA.

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10) in both AOH and sham-cannulated rats suggests that this transitory effect is due to the inflammatory response triggered by the cannulation procedure itself. However, the GCL density in AOH rats shows a ~70% reduction in numbers. Complementing the structural changes, the functional measurement of RGC (in terms of pSTR) and bipolar cell (in terms of scotopic b-wave) responses also showed a reduction (~50%) with little or no recovery at week 4. The a-wave response, which is related to the outer retinal layer, remained grossly normal. The observation of differential dysfunction of inner and outer retinal responses with a poor recovery of inner retinal component (pSTR) in the present study is in agreement with the findings of Bui et al. Those authors also reported a partial recovery of pSTR from IOP

![Figure 6](image-url)
elevation of 70 mm Hg to no recovery if IOP peaked at 100 mm Hg for 105 minutes.

Under pretreatment conditions, both the LBP dosages (1 and 10 mg/kg LBP) delayed the onset and slowed down the rate of IRL thinning as well as preserving the inner retinal function. The percentage at which the LBP 1 mg/kg retained the inner retinal thickness at day 10 was comparable to results from another report on a mouse AOH model (90 mm Hg for 60 minutes) at day 7. As the procedure of the cannulation alone was known to induce transient RNFL thickening, the measurements including RNFL may overestimate the IRL thickness. Therefore, the present study further differentiated the IRL thickness into RNFL and IPL + INL to minimize the chance of overstating the protective effect of LBP. Furthermore, both LBP dosages equally preserved the GCL density in AOH rats to the level of sham cannulation at week 4. He et al., using the same rat strain (SD rats) as the present study, showed that pretreatment with LBP 1 mg/kg preserved only 50% of RGC in AOH rats (130 mm Hg for 60 minutes) compared with 30% RGC survival in the PBS-treated group at day 7. The direct comparison of the neuroprotective outcome between these studies has certain limitations. The differences in the selection of IOP, duration of AOH, methods of investigation, and time of assessment between the studies are likely to account for the differential rate of structural loss or various neuroprotective effects being reported. As the present study did not evaluate the rate of RGC loss at the end of week 1, the OCT-measured IRLT (sum of IPL and INL) has to be

<table>
<thead>
<tr>
<th>Experimental Groups, N</th>
<th>AoH-Vehicle Control, Pretreated, n = 9</th>
<th>AoH-LBP 1 mg/kg, Pretreated, n = 8</th>
<th>AoH-LBP 10 mg/kg, Pretreated, n = 13</th>
<th>AoH-LBP 10 mg/kg, Posttreated, n = 8</th>
<th>Sham Control, n = 6</th>
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</thead>
<tbody>
<tr>
<td>pSTR, mean (SEM), ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>113.9 (4.2)</td>
<td>118.3 (4.5)</td>
<td>113.7 (3.5)</td>
<td>122.5 (4.5)</td>
<td>121.0 (5.2)</td>
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<tr>
<td>Day 10</td>
<td>122.8 (4.9)</td>
<td>118.7 (5.2)</td>
<td>114.5 (4.1)</td>
<td>124.1 (5.2)</td>
<td>115.8 (6.0)</td>
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<tr>
<td>Day 28</td>
<td>127.3 (4.4)</td>
<td>119.9 (3.7)</td>
<td>119.5 (4.7)</td>
<td>131.4 (4.7)</td>
<td>116.4 (5.4)</td>
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<tr>
<td>b-wave, mean (SEM), ms</td>
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<td></td>
<td></td>
<td></td>
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<td>Baseline</td>
<td>68.2 (3.2)</td>
<td>75.7 (3.4)</td>
<td>73.2 (2.7)</td>
<td>72.7 (3.4)</td>
<td>75.1 (3.9)</td>
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<tr>
<td>Day 10</td>
<td>81.0 (4.8)</td>
<td>83.4 (5.1)</td>
<td>81.8 (4.0)</td>
<td>78.2 (5.1)</td>
<td>84.4 (5.9)</td>
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<td>Day 28</td>
<td>78.5 (5.0)</td>
<td>74.5 (5.3)</td>
<td>73.7 (4.1)</td>
<td>80.6 (5.3)</td>
<td>81.9 (6.1)</td>
</tr>
<tr>
<td>a-wave, mean (SEM), ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>8.0 (0.1)</td>
<td>7.9 (0.1)</td>
<td>8.0 (0.1)</td>
<td>7.9 (0.1)</td>
<td>8.1 (0.1)</td>
</tr>
<tr>
<td>Day 10</td>
<td>8.3 (0.1)</td>
<td>8.0 (0.2)</td>
<td>8.1 (0.1)</td>
<td>7.8 (0.2)</td>
<td>8.2 (0.2)</td>
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<tr>
<td>Day 28</td>
<td>8.0 (0.2)</td>
<td>8.0 (0.2)</td>
<td>8.2 (0.1)</td>
<td>8.6 (0.2)*†</td>
<td>7.9 (0.2)</td>
</tr>
</tbody>
</table>

* P < 0.001 when compared to baseline.
† P < 0.01 when compared to day 10.

![Figure 7](image_url)
Posttreatment Neuronal Rescue Effect of LBP

In summary, the present study differentiates the posttreatment outcome, that is, slowing down secondary degeneration and improving the retinal function, LBP may be a prospective treatment option for patients with the above diseases.

While LBP therapy showed promising outcomes in preclinical animal trials, its effect on patients with ocular diseases remained largely unknown. Recently, a double-masked clinical trial showed that daily supplements of Lycium barbarum over 12 months in retinitis pigmentosa patients preserved macular thickness and visual acuity, thus exhibiting a cone rescue effect. Even before any clinical study could be planned to test the neuroprotective effect of LBP on glaucoma patients, extensive preclinical studies are warranted to investigate the therapeutic efficacy of LBP posttreatments in experimental models of chronic glaucoma, with and without an IOP-lowering effect.

In summary, the present study differentiates the posttreatment efficacy of LBP from pretreatment outcomes using an AOH rat model. Under pretreatment conditions, the structural and functional rescue effects observed earlier in the course of study resulted in longitudinal preservation. In contrast, the

should be noted that the cell count computed from the H&E-stained GCL layer contains a comparable number of RGC and displaced amacrine cells. Sun et al. showed that ~65% of H&E-stained cells were identified as RGC (immunostained with gamma synuclein) in both normal and optic nerve-crushed eyes of Sprague-Dawley rats. Further, both types of staining were able to reflect a similar percentage of RGC loss (~50%) following the insult (day 14), which approximated the RGC loss (~65%) quantified using anterograde tracer cholera toxin subunit B. Although the present study did not use any specific biomarkers for RGC labeling, we speculated that the pre- and posttreatment were able to achieve effective RGC rescue, as the GCL densities of sham and LBP-treated rats were similar.

An initial study investigating the dose response regarding LBP under pretreatment conditions, using an ocular hypertension rat model (induced by laser photocoagulation), revealed that 1 and 10 mg/kg LBP offered similar therapeutic effects.22 Later studies applied LBP 1 mg/kg to test the neuroprotective effects using variants of optic neuropathy.23–29 However, another study concluded that pretreatment with LBP 10 mg/kg offered relatively better neuroprotective effects than 1 mg/kg in an experimental stroke model.13 Thus, in this study, a dosage of LBP 10 mg/kg was chosen to investigate the neuroprotective effects under pre- and posttreatment conditions using combined structure–function tools as compared to the more commonly trialed dosage of pretreatment LBP 1 mg/kg. As the results could show the neuronal rescue effect of LBP under posttreatment condition, future studies should explore the dose response with LBP introduced at later time points (or) at different stages of the disease condition.

Previous studies have reported that LBP pretreatment in an AOH model activated the Nrf2/ HO-1 antioxidant pathway by upregulation of nuclear factor erythroid 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) expression to combat the effects of oxidative stress.26 LBP also protected the retinal vasculature by downregulating the expression of endothelin-1, advanced glycation end products (AGE), receptor of AGE, and amyloid-β protein, thus maintaining the integrity of the blood–retinal barrier.29 As the mechanism of neuroprotection in the model resulted in longitudinal preservation. In contrast, the
rescue effect with posttreatment was not evident initially; it subsequently slowed down secondary degeneration along with the improvement in function. Although pretreatment with LBP offers relatively better neuroprotection, the beneficial effect of posttreatment is still remarkable as compared with the untreated condition.

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