



Published in final edited form as:

Neurosci Lett. 2018 March 23; 670: 89–93. doi:10.1016/j.neulet.2018.01.043.

The effect of celastrol on the ocular hypertension-induced degeneration of retinal ganglion cells

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Abstract

Celastrol, a quinone methide triterpene extracted from the perennial vine *Tripterygium wilfordii*, has been identified as a neuroprotective agent in various models of neurodegenerative disorders. We have reported earlier that systemic and intravitreal administration of celastrol stimulate the survival of retinal ganglion cells (RGCs) injured by optic nerve crush (ONC) and that mechanisms underlying celastrol's RGC protection may be associated with inhibition of TNF- α -mediated cell death. The present study evaluates the effect of celastrol on the survival of RGCs injured by ocular hypertension. Intraocular pressure (IOP) elevation resulted in approximately 23% of RGCs loss. Reduction in RGC numbers was observed in all four retinal quadrants: 30% in superior, 17% in inferior, 11% in nasal and 35% in temporal regions. Celastrol (1 mg/kg) or vehicle (DMSO) was administered three times per week by intraperitoneal injection, starting on the day of laser photocoagulation of the TM and continued for the entire duration of the experiment (5 weeks). Celastrol treatment stimulated RGC survival by an average of 24% in the entire retina compared to the vehicle-treated group. RGC numbers were increased in all four quadrants: approximately 40%, 17%, 15% and 30% more RGCs were counted in the superior, inferior, nasal and temporal regions, respectively. The average RGC numbers for the entire retinas of the celastrol/IOP group were only ~5% and 10% lower than that in vehicle- or celastrol-injected animals with normal IOP, respectively. Our data indicate a significant celastrol-mediated neuroprotection against elevated IOP-induced injury.

Keywords

Celastrol; Retina; Ganglion cells; Optic nerve; Glaucoma; Neuroprotection

1. Introduction

Glaucomatous neuropathy affects millions of people worldwide and is often only diagnosed after patients have already suffered irreversible damage to their vision. If left untreated, glaucoma could lead to debilitating visual impairments. Vision loss in this disease is due to damage to retinal ganglion cells (RGCs) and their axons in the optic nerve. The current

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reduced, suggesting that celastrol-mediated protection of ONC-injured RGCs involves cell death pathways associated with TNF- α .

The current study evaluates the effect of celastrol on the survival of RGCs in an ocular hypertensive animal model of RGC degeneration. Both ONC and elevated IOP models are characterized by progressive degeneration of RGCs, however the IOP model is traditionally considered to be more relevant to human glaucoma since the elevated IOP is a well recognized risk factor for the most common forms of the disease.

2. Materials and methods

2.1. Animals

Adult Brown Norway rats (3 month-old; 250–300 g) were used to generate experimental glaucoma models. The use of animals and the procedures involving animals were approved by the Animal Research Committee of the University of California at Los Angeles and were in compliance with the National Institutes of Health Guide for the Care and Use of Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed with standard food and water provided ad libitum in a room with the temperature set at 21 °C and illuminated with fluorescent lights (330 lx) automatically turned on at 03:00 am and off at 03:00 pm. The animals were kept at least 1 week in this environment before IOP measurement or trabecular meshwork (TM) laser photocoagulation. Photocoagulation was performed on one eye of each rat while the contralateral eye served as an untreated control. Celastrol (1 mg/kg) or vehicle (DMSO) was administered three times per week by i.p. injection. Four groups of animals were used in this study to evaluate celastrol's cell protective effect: Vehicle (untreated eyes of animals injected with vehicle; n = 10), Vehicle/IOP (laser-treated eyes of animals injected with vehicle; n = 10), Celastrol (untreated eyes of animals injected with celastrol; n = 14), and Celastrol/IOP (laser-treated eyes of animals injected with celastrol; n = 14).

2.2. Ocular hypertension model

Trabecular laser photocoagulation was used to increase IOP. After 1 week of accommodation, light and dark phase IOPs were measured in awake Brown Norway rats with Tonolab (TonoLab; Colonial Medical Supply, Franconia, NH) twice a week. Trabecular laser photocoagulation was performed as described previously (Kwong et al., 2011). Briefly, approximately 200 laser burns were delivered ab externo to the 360° TM at laser settings of 200 μ m diameter, 100 mW power, and 50 m sec durations. Trabecular laser photocoagulation was repeated 2 weeks after the first treatment on eyes with no significant IOP elevation compared with contralateral control eyes. IOP elevation was sustained for 5 weeks.

2.3. Quantification of RGCs

Animals were deeply anesthetized with intramuscular injections of 80 mg/kg sodium pentobarbital. The eyes were enucleated, immersed in fixative for 0.5 h, and the lenses were removed. The eyecups were postfixed for another 0.5 h. Entire retinas were used for immunohistochemistry with a custom-made antibody against Rbpms according to a

published protocol [20]. Briefly, the retinas were washed with PBS, incubated with 10% fetal bovine serum for 1 h to block nonspecific staining, followed by incubation with the primary antibody against Rbpms in PBS containing 1% triton, 0.5% BSA, and 0.9% sodium chloride (PBS-T-BSA) overnight at 4 °C. The retinas were washed in PBS-T-BSA and incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG antibody (1/1000) overnight at 4 °C. Retinas were then washed with PBS, flat mounted with several radial cuts on a glass slide, air dried and covered with a cover-slip in an aqueous mount. Topographical analysis of RGC density was performed under a fluorescence microscope (LSM410; Carl Zeiss, Oberkochen, Germany). Three areas (0.32 × 0.24 mm each) per retinal quadrant (superior, temporal, inferior and nasal) at 1, 2, 3 and 4 mm from the optic disc were analyzed. RGCs were counted in a masked manner.

2.4. Statistical analysis

Data are presented as the mean ± standard deviation (SD). A repeated measures ANOVA was conducted to compare RGC density in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups. All measurements at four distances and four quadrants from each animal were included in the overall ANOVA model comparing RGC density in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups, and the effects of quadrants and distances were controlled by multi-factors ANOVA model. When comparisons of RGC density among groups were performed within each quadrant, all measurements at four distances from each animal were included in the ANOVA model and the distance effect was controlled by multi-factors ANOVA model. $P < 0.05$ was considered statistically significant.

3. Results and discussion

The rat experimental glaucoma model to investigate celastrol's cell protective effect against ocular hypertension induced RGC damage was generated by laser photocoagulation of the TM. Mean IOPs for the light and dark phases of the circadian cycle for experimental and control eyes were calculated and representative IOP profiles of vehicle-treated rat and celastrol-treated rat are shown in Fig. 1A and B respectively. TM photocoagulation to elevate IOP was performed unilaterally, while the contralateral eye received no treatment. The average light phase IOP in the Vehicle/IOP and Celastrol/IOP groups were 23.81 ± 2.68 mmHg and 21.74 ± 3.13 mmHg, respectively compared to 18.91 ± 1.07 in Vehicle group and 17.81 ± 1.06 mmHg in Celastrol group. The dark phase average IOP values were 32.67 ± 0.99 , 32.15 ± 1.56 , 41.17 ± 4.63 and 39.52 ± 4.99 mmHg in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups, respectively. As expected, both the light and dark phase IOP in experimental eyes of vehicle-treated animals were significantly elevated after TM photocoagulation, when compared to contralateral eyes (light IOP: $P = 0.0002$; dark IOP $P = 0.0002$). Similarly, both the light and dark phase IOP in experimental eyes of celastrol-treated animals were significantly elevated after TM photo-coagulation, when compared to contralateral eyes (light IOP: $P = 0.0004$; dark IOP $P = 0.0001$). There was no statistical difference between the average light and dark IOP phases of experimental eyes from celastrol-treated group and vehicle-treated group (light IOP: $P = 0.1$; dark IOP: $P = 0.41$). Since a single laser TM photocoagulation was insufficient to maintain elevated IOP for the duration of the experiment, a second treatment was performed two weeks after the first

procedure. Although the first and second TM photocoagulation both resulted in a significant response of the light phase IOP, elevation of the dark phase IOP in experimental eyes was more prominent after the second laser procedure.

To evaluate the cell protective effect of celastrol, animals were treated i.p. with 1 mg/kg of the drug three times per week starting on the day of laser photocoagulation of the TM and continued for the entire duration of the experiment (5 weeks). The control rats received the vehicle (DMSO). The frequency of the drug administration in this study was reduced from daily i.p. injections of 1 mg/kg of celastrol that was used in experiments with ONC animals to avoid its potential side effects. The regimen for celastrol treatment in experiments with the ONC model was based on earlier published studies on successful use of celastrol in rat and mouse models of various human diseases with no side effects [21–23]. However, we observed an approximately 2% body weight loss in the celastrol-injected animals, whereas animals in the control group injected with DMSO only registered a weight gain of 6% during a two-week period [19]. Consistent with our findings, the body weight decrease of animals treated with celastrol was also observed during i.p. treatment of six-week-old diabetic db/db and non-diabetic db/m mice with 1 mg/kg/day of celastrol for two weeks [23]. No side effects, including the effect on the weight of animals, from the celastrol regimen used in this study, were observed.

The effect of celastrol on RGC survival was determined by comparing the density of Rbpm-labeled RGCs in flat mounted retinas with and without induced ocular hypertension in inferior, nasal, superior and temporal retinal quadrants of control (Vehicle group) and experimental animals (Celastrol group). All measurements at four distances and four quadrants from each animal were included in the overall ANOVA model comparing RGC density in Vehicle, Celastrol, Vehicle/IOP and Celastrol/IOP groups, and the effects of quadrants and distances were controlled by multi-factors ANOVA model. When comparisons of RGC density among groups were performed within each quadrant, all measurements at four distances from each animal were included in the ANOVA model and the distance effect was controlled by multi-factors ANOVA model. The average number of RGCs in the entire retinas of DMSO- and celastrol-treated animals was similar: 1836 ± 562 cells/mm² in the Vehicle group and 1949 ± 594 cells/mm² in the Celastrol group. Vehicle-injected animals with ocular hypertension suffered a loss of approximately 23% of RGCs compared to vehicle injected animals with normal IOP [Veh vs Veh/IOP, $P = 0.0078$]. Reduction in RGC numbers was observed in all four retinal quadrants analyzed in this study: 30% in superior ($P = 0.051$), 17% in inferior ($P = 0.001$), 11% in nasal ($P = 0.11$) and 35% in temporal ($P = 0.012$) regions, although RGC loss in only inferior and temporal regions was statistically significant (Fig. 2, Table 1). Uneven RGC damage associated with IOP elevation in this animal model may be related to the well-characterized focal retinal neurodegeneration in human glaucoma. In animals treated with celastrol, the effect of IOP elevation was much less dramatic: celastrol-mediated support for RGC survival in the entire retina resulted in preservation of approximately 24% more cells compared to that of the vehicle-treated group (Cel/IOP vs Veh/IOP, $P = 0.021$; Figs. 2 and 3). RGC protection was observed in all four quadrants: ~40% ($P = 0.044$) more RGCs were counted in the superior region, 17% more in inferior ($P = 0.0041$), 15% more in nasal ($P = 0.038$) and 30% more in temporal ($P = 0.12$; Fig. 2). The average number of RGC in the entire retinas of the

celastrol/IOP group were about 5% and 10% lower compared to vehicle- and celastrol-treated animals with normal ocular tensions, respectively. With respect to potential underlying mechanisms in celastrol-mediated RGC protection, earlier, we had shown that celastrol's protective effect on RGCs injured by ONC may be associated with the downregulation of TNF- α . Since TNF- α signaling has been implicated in glaucomatous neurodegeneration [24], it is possible that the RGC protective effect of celastrol from IOP-induced injury also involves modulation of TNF- α expression.

In summary, the celastrol-mediated support for RGC survival in the animal model of ocular hypertension described in this paper, together with our earlier published data on RGC protective effect of this drug against optic nerve crush injury, provide support for the further evaluation of celastrol as potential treatment for glaucoma and other optic neuropathies.

Acknowledgments

Funding

This work was supported by The Research to Prevent Blindness (JC) and by the National Institutes of Health (NIH) Grant EY018644 (NP). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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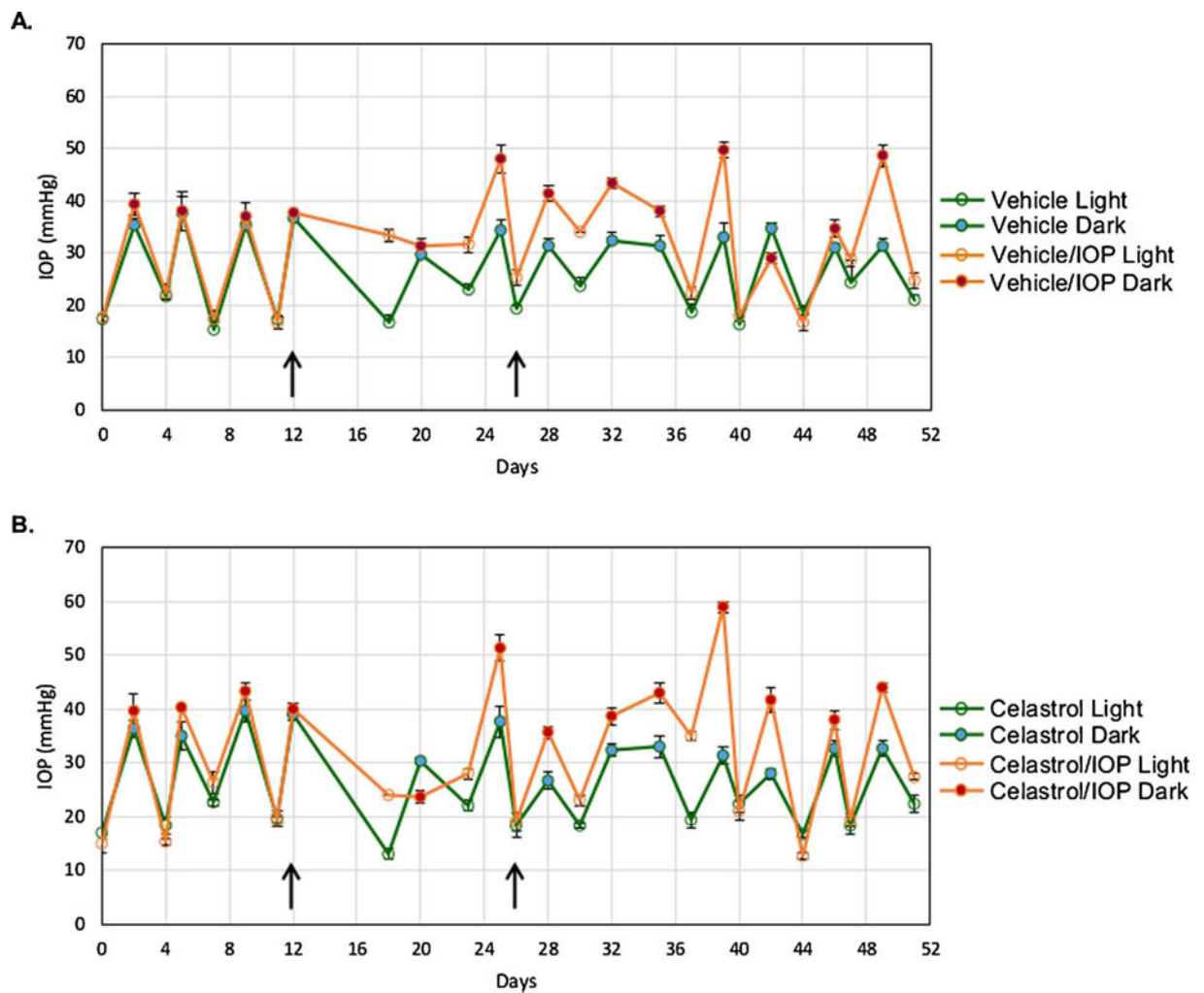


Fig. 1. Representative IOP profiles for vehicle- (A) and celastrol-treated (B) animals. The light and dark phase IOP readings of an individual rat from the vehicle-treated group (Animal #2334) and celastrol-treated group (Animal #2325) are shown. One IOP reading was recorded by TonoLab after five successful measurements were made. Three IOP readings were used for IOP analysis in this experiment. Data are presented as mean \pm SD. The laser trabecular meshwork photocoagulation indicated by arrows was performed unilaterally, twelve days after establishing the baseline IOP, and was repeated two weeks later to maintain elevated IOP during the course of experiment. The contralateral eyes received no treatment and served as controls.

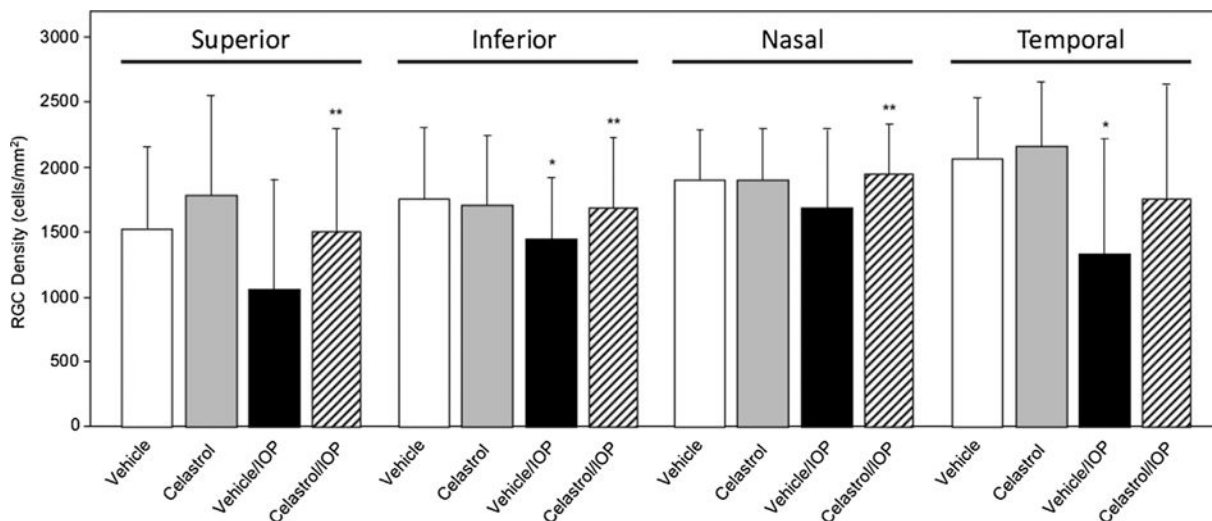


Fig. 2.

The effect of celastrol treatment on RGC numbers in superior, inferior, nasal and temporal retinal quadrants. A repeated measures ANOVA was conducted to compare RGC density in Vehicle, Celastrol, Vehicle/IOP, and Celastrol/IOP groups. A significant decrease in RGC density in Vehicle/IOP compared to Vehicle group was observed in the inferior ($*P = 0.001$) and temporal ($*P = 0.012$) quadrants. RGC density was significantly increased in the superior ($**P = 0.044$), inferior ($**P = 0.0041$) and nasal ($**P = 0.038$) retinal quadrants of Celastrol/IOP compared to Vehicle/IOP animals. The effect of distance (mm) from the optic nerve head was adjusted. Data are presented as mean \pm SD.

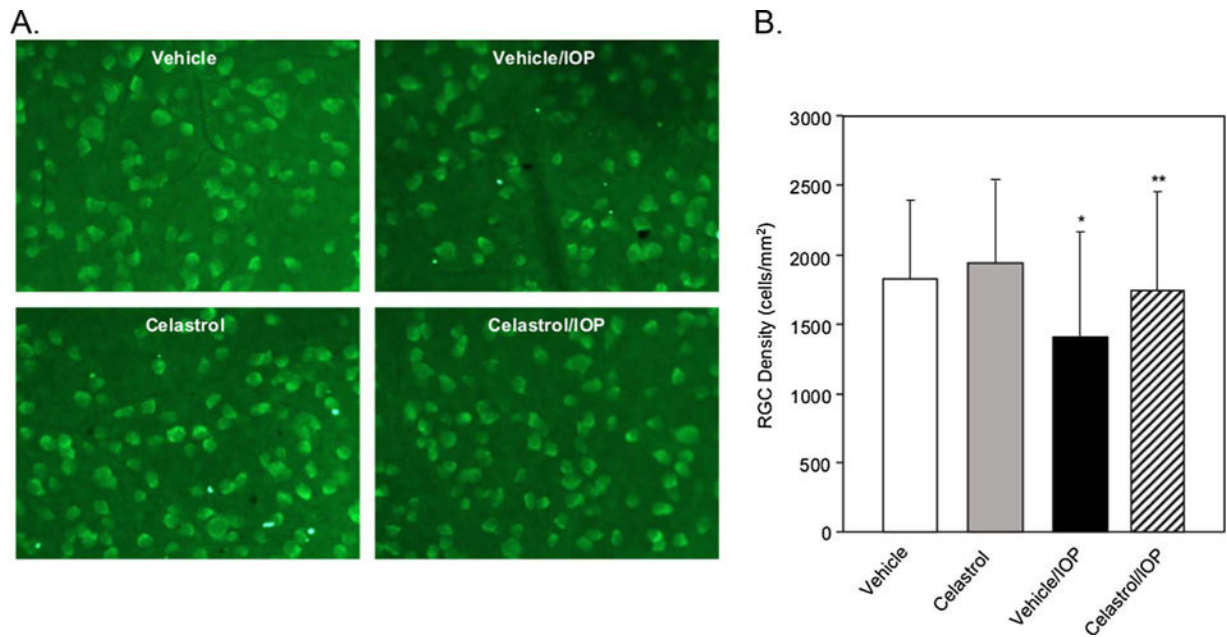


Fig. 3. Celastrol supports RGC survival from ocular hypertension damage. **A.** Rbpm5-positive RGCs in flat mounted retinas from vehicle- and celastrol-treated animals with normal and elevated IOP. Images represent temporal retinal quadrants. **B.** A repeated measures ANOVA was conducted to compare RGC density in vehicle-treated, celastrol-treated, vehicle-treated with IOP elevation, and celastrol-treated with IOP elevation groups after controlling for the quadrant and distance effects. A significant decrease of RGC density was observed in Vehicle/ IOP compared to Vehicle group (* $P=0.0078$). There was a significant increase of RGC density in Celastrol/IOP compared to Vehicle/IOP group (** $P=0.021$). Data are presented as mean \pm SD.

Table 1 Retinal ganglion cell density in superior, inferior, nasal and temporal retinal quadrants in animals treated with celastrol or vehicle. Data presented as mean ± SD.

Animal Group	Distance from ONH															
	Inferior				Nasal				Superior				Temporal			
	1 mm	2 mm	3 mm	4 mm	1 mm	2 mm	3 mm	4 mm	1 mm	2 mm	3 mm	4 mm	1 mm	2 mm	3 mm	4 mm
Vehicle	2204.5 ± 369.4	2237.2 ± 281.3	1665.8 ± 174.5	1014.6 ± 145.1	2246.3 ± 311.5	2116.9 ± 324.3	1875.0 ± 216.6	1474.9 ± 233.7	2330.0 ± 276.1	1712.9 ± 335.8	1248.7 ± 450.0	889.1 ± 355.8	2247.6 ± 315.3	2387.6 ± 324.4	2314.3 ± 269.4	1410.8 ± 193.5
Celastrol	2246.2 ± 209.2	2076.2 ± 254.6	1606.4 ± 293.6	1006.8 ± 273.5	2353.6 ± 135.9	2282.6 ± 195.0	2065.0 ± 201.2	1509.3 ± 265.8	2595.4 ± 245.4	2341.4 ± 511.3	1367.3 ± 351.0	933.0 ± 284.8	2455.4 ± 287.3	2412.4 ± 281.7	2381.6 ± 304.3	1550.4 ± 390.0
Vehicle/IOP	1950.8 ± 166.2	1736.4 ± 239.2	1375.5 ± 244.8	826.4 ± 134.3	1987.4 ± 483.4	1965.2 ± 655.3	1701.1 ± 595.2	1216.0 ± 459.7	1354.6 ± 1021.4	1417.4 ± 949.3	1036.9 ± 745.7	538.7 ± 370.3	1400.4 ± 958.2	1503.7 ± 973.5	1480.1 ± 981.1	1070.9 ± 748.8
Celastrol/IOP	2195.7 ± 223.3	2017.3 ± 266.4	1631.6 ± 377.5	1027.3 ± 372.3	2209.7 ± 256.3	2190.1 ± 257.4	2036.9 ± 256.8	1448.6 ± 295.4	1786.6 ± 893.1	1978.1 ± 774.3	1451.4 ± 618.4	887.3 ± 496.3	1886.6 ± 934.2	2035.1 ± 968.3	1919.3 ± 894.4	1264.6 ± 668.9