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Morphological changes and potential mechanisms of intraocular pressure reduction after micropulse transscleral cyclophotocoagulation in rabbits

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\textbf{Short Title:} Mechanism of MP-TSCPC in rabbits

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\textbf{Keywords:} micropulse transscleral cyclophotocoagulation; intraocular pressure; ciliary body; mechanism; rabbit
Abstract

Introduction: Micropulse transscleral cyclophotocoagulation (MP-TSCPC) is a method for intraocular pressure (IOP) reduction in patients with glaucoma; however, the specific mechanisms underlying its ability to reduce IOP remain unclear. We therefore investigated the morphological changes and mechanisms of intraocular pressure (IOP) reduction after MP-TSCPC.

Methods: The right eyes of four pigmented rabbits were treated with MP-TSCPC with power setting corresponding to those used in glaucoma patients (1 power: 2,000 mW; time: 160 sec; duty cycle: 31.3%). Power settings of 1, 1/8, 1/16, and 1/32 power were applied to the right eyes. The left eyes were used as controls. A light microscope and electron microscope were used to observe morphological findings after 1 week of MP-TSCPC. IOP and IOP reduction rate were compared before and after MP-TSCPC application on days 1, 3, and 5, and at 1 week.

Results: In the pre-MP-TSCPC, IOP was 16.7 ± 0.6 mmHg. The IOP of rabbit treated with the 1 power was 3 mmHg, with an IOP reduction rate of 80%; however, the eyes developed phthisis bulbi. The IOP was 7.0 ± 0.0 mmHg 1 week after MP-TSCPC (IOP reduction rate: 59%) in rabbit treated with the 1/8 power. Reduction in IOP was observed, but there was significant tissue invasion to the ciliary body. The IOP was 10.3 ± 0.6 mmHg (IOP reduction rate: 40%) 1 week after MP-TSCPC in rabbit treated with the 1/16 power, which was more effective to reduce IOP than that with the 1/8 power. Tissue invasion to the ciliary body was negligible, nonpigmented epithelial cells of the pars plicata were damaged, basal infoldings were destroyed, and repair was accompanied by proliferating tissue. No IOP reduction or tissue change was observed in rabbit treated with the 1/32 power.

Conclusion: A potential mechanism for IOP reduction in pigmented rabbits is aqueous humor transport dysfunction due to damage to the nonpigmented epithelial cells of the pars plicata and destruction of basal infoldings. The power of MP-TSCPC was consistent with both morphological changes and IOP reduction.
Introduction

Techniques for destruction of the ciliary body to reduce IOP include diathermic coagulation [1] developed in the 1930s, cyclocoagulation [2] developed in the 1950s, and photocoagulation developed in the 1970s. Photocoagulation includes transpupillary photocoagulation [3], which uses an argon laser, and transscleral photocoagulation, which uses a diode laser [4] of 810-μm wavelength and Nd:YAG laser [5] of 1064-μm wavelength. The purpose of using these techniques is to destroy the pars plicata, causing aqueous humor transport dysfunction and IOP reduction [6]. However, as serious complications including pain during treatment and phthisis bulbi after treatment may occur [7], they are considered the last resort for intractable glaucoma, in which effective IOP reduction cannot be attained by medication or surgery [8].

Micropulse transscleral cyclophotocoagulation (MP-TSCPC) uses diode laser photocoagulation of 810-μm wavelength (Cyclo G6™, Iridex) and MP3 probes. This technique was recently approved in Japan in October 2017 (approved by the FDA in 2015) [9]. An advantage of MP-TSCPC is that this method can be used not only for refractory glaucoma but also for glaucoma of any stage. Repetitive treatment is possible as continuous oscillations between the on and off states of the laser make it difficult for the temperature of surrounding tissue to rise.

With regards to the effectiveness of IOP reduction, the reduction rate is approximately 26% to 45% [10-14]. Aquino et al. [15] compared conventional photocoagulation (continuous wave-transscleral cyclophotocoagulation [CW-TSCPC]) with MP-TSCPC and defined success as follows: IOP after treatment is 6–21 mmHg and the IOP reduction rate is 30% or more. Eighteen months after treatment, the success rate in the MC-TSCPC group did not statistically differ from that in the CW-CPC group (52% vs. 30%, P = 0.13); however, ocular complications, such as anterior chamber inflammation, phthisis bulbi, and pain after treatment, were observed much less in the MC-TSCPC group (P = 0.01). These previous findings indicate that MC-TSCPC is both a more effective and safer method than CW-CPC. The underpinning mechanism is destruction of the pars plicata by CW-TSCPC via a continuous wave [16-18], whereas MP-TSCPC is believed to involve an expansion of the uveoscleral pathway by constriction of ciliary muscles [19] and aqueous humor dysfunction [9,20] induced by stimuli from pars plicata to pars plana. However, the specific mechanism remains unclear.

To address this, we aimed to compare the IOP and IOP reduction before and after treatment in pigmented rabbits and to perform morphological observations to clarify the specific mechanisms underpinning IOP reduction by MP-TSCPC.
Materials and Methods

**Animals**
Eight eyes of four Dutch rabbits of 20–44 weeks old (29.5 ± 9.0, mean ± standard deviation), weighing 1.8–2.6 (2.1 ± 0.3) kg were used. All protocols were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with the approval of the Animal Experiment Ethics Committee of Kitasato University (approval number: 2018-183). An anesthetic mixture of Domitor (medetomidine hydrochloride) 0.25 mL/kg, Vetorphale 0.5 mg/kg, and midazolam 0.5 mg/kg was administered intramuscularly. All experiments were conducted under anesthesia.

**Treatment methods and conditions**
We used Cyclo G6™ and MP3 probe to emit micro pulse waves to the whole circumference of the ciliary body at 1.5 mm of cornea limbus, eliminating the areas of the 3 and 9 o’clock directions on the conjunctiva. The right eyes were treated, and the left eyes were used as controls. After emission, 0.5% levofloxacin and 0.1% bromfenac eye drops were applied. There are reports that MP-TSCPC have irradiated rabbits [21], but Table 1 shows the powers used that are consistent with treatment conditions. Power settings corresponding to those used in human glaucoma patients were used in rabbits as follows: 2,000 mW, time 160 sec, duty cycle 31.3% (1 power); 1,000 mW, time 40 sec, duty cycle 31.3% (1/8 power); 500 mW, time 40 sec, duty cycle 31.3% (1/16 power); and 250 mW, time 40 sec, duty cycle 31.3% (1/32 power).

**Measuring IOP**
Tonovet tonometer (Tonovet®, ICARE, Finland OY) [22] was used, and the mean value was derived from the values measured 1 week before treatment, which was defined as the pre-IOP. The mean value and standard deviation were derived from three measurements obtained on days 1, 3, and 5, and at 1 week. The measuring time was always the same (2 PM), considering daily variation in IOP of rabbits [23]. According to a previous report [15], the definition of effective IOP reduction after treatment was as follows: ≥6 mmHg and ≥30% of IOP reduction compared to the pre-IOP.

**Dissecting, light, and electron microscopy**
For the ciliary body, the isolated eyes of rabbits were immersed immediately in 2.5% glutaraldehyde and buffered with 0.1 M phosphate solution (pH 7.4) overnight and halved into
equatorial segments. The whole inner ciliary body was observed with a dissecting microscope (SMZ-10; Nikon, Tokyo, Japan). Then, the ciliary body was dissected into small pieces and immersed in 1% OsO₄ for post fixation. For dehydration, 50, 70, 80, 90, 95, and 100% (absolute) ethanol was used. Next, the pieces were displaced with n-Butyl glycidyl ether, embedded in Quetol resin, and thermally polymerized. A glass knife was made to cut the resin block into 200 nm thickness by an ultramicrotome. The cut sections were then stained with toluidine blue and observed under a light microscope (BX53; Olympus, Tokyo, Japan). The tissue was cut into 80 nm thick sections, double stained using lead citrate and uranium acetate, and observed under an electron microscope (H-7600; Hitachi, Tokyo, Japan).

The ciliary body for histochemistry was cold formalin-fixed and dissected into small pieces for quick freezing for embedding. A cryostat was used to create frozen sections, and the tissues were filtrated before usage and immersed in the substrate solution of compound liquid consisting of 0.125% ATP-2Na, 0.2 MTris-HCL buffer (pH7.2), 0.1 M magnesium sulfate, and 2% lead nitrate at 37°C for 30 minutes. The tissues were then washed with distilled water, and mitochondria ATPase was detected by metallic salt reaction with 1% ammonium sulfide solution. The conjunctiva was formalin-fixed (SuperFix®; Kurabo, Osaka, Japan), and paraffin blocks were made. Then, the blocks were cut into slices of 4 µm and stained with hematoxylin-eosin.

Observations with light and electron microscopy are highly subjective. Therefore, two authors (TT and KA) observed the sections with the light microscope, two other authors (SU and KM) observed the sections with the electron microscope, and then all the co-authors judged the morphological findings.

**Results**

**IOP progress**

**Figure 1** shows the results of IOP. The rabbit treated with power settings corresponding to that of humans (1 power) had pre-IOP of 16.7 ± 0.6 mmHg, which reached 3.3 ± 0.6 mmHg the following day of treatment (IOP reduction rate: 80%). The IOP remained low from that timepoint and shifted to low IOP of about 3 mmHg (approximately 82%). IOP did not recover, and the eyes were isolated after 1 week. IOP of the rabbit treated with 1/8 power setting (1/8 power) was 17.0 ± 1.0 mmHg before treatment, 13.3 ± 2.5 mmHg (22%) after 1 day, 7.7 ± 0.6 mmHg (55%) after 3 days, 6.0 ± 1.0 mmHg (65%) after 5 days, and 7.0 ± 0.0 mmHg (59%) after 1 week. Effective IOP reduction was identified, and the eyes were isolated. IOP of the
rabbit treated with 1/16 power setting (1/16 power) was 17.3 ± 0.6 mmHg before treatment, 11.3 ± 1.5 mmHg (35%) after 1 day, 11.3 ± 0.6 mmHg (35%) after 3 days, 8.7 ± 0.6 mmHg (50%) after 5 days, and 10.3 ± 0.6 mmHg (40%) after 1 week. The IOP reduction rate was lower than that of the rabbit treated with 1/8 power, but effective IOP reduction was observed, and the eyes were isolated 1 week after the treatment. IOP of the rabbit treated with 1/32 power setting (1/32 power) was 16.7 ± 0.6 mmHg before treatment, 15.3 ± 1.1 mmHg (8%) after 1 day, 16.7 ± 0.6 mmHg (0%) after 3 days, 15.7 ± 0.6 mmHg (5%) after 5 days, and 17.3 ± 0.6 mmHg (4% rise) after 1 week. No IOP reduction was identified, and the eyes were isolated 1 week after the treatment.

**Dissecting microscopy**

Compared to the control eye (Figure 2A), the 1 power had severe conjunctival chemosis, damaged cornea epithelium, and hyphema the day after treatment, which did not recover. Phacoysis as well as ciliary body and retina hemorrhage were observed, and phthisis bulbi developed (data not shown). The 1/8 power did not exhibit conjunctival chemosis, damaged cornea epithelium, or paralytic mydriasis at any stage. The anterior chamber did not have severe inflammation such as fibrin deposition. Unarranged pars plicata and hemorrhage from folds were observed, but no conjunctival scar on the treated section or thinning of the sclera was observed (Figure 2B). The 1/16 power did not have any complications at any stage. The conjunctiva and sclera were normal, and no hemorrhage was observed even though the entire pars plicata was swollen (Figure 2C). The 1/32 power was normal, although a portion of pars plicata was narrow (Figure 2D).

**Light microscopy**

The pars plana did not exhibit any tissue changes, so we focused on changes in pars plicata. Compared to the control eye (Figure 3A), the 1 power had phthisis bulbi; therefore, pars plicata could not be collected. The 1/8 power had enlarged ends of pars plicata, damaged tissue, and hemorrhage. Further, capillary vessels of the ciliary body disappeared (Figure 3B). The 1/16 power had inflected and folded pars plicata were observed (Figure 3C). The 1/32 power only exhibited inflected pars plicata, and other tissue findings were normal (Figure 3D).

We evaluated mitochondrial ATP activity in the 1/16 and 1/32 power whose tissue changes were relatively minor, by metallic salt reaction (adenosine triphosphatase [ATPase]). Compared to the control eye (Figure 4A), the 1/16 power exhibited disturbances associated with destroyed
basal infoldings of pars plicata but had the ATP activity as that of the control eye (Figure 4B). The 1/32 power had normal basal infoldings of pars plicata without any damage (Figure 4C).

The area of treated conjunctiva was compared to that of the controls (Figure 5A). The 1/16 power had damage only in stratified squamous epithelial cells (Figure 5B). The 1/32 power did not exhibit any damage (Figure 5C).

**Electron microscopy**

Compared to the control eye (Figure 6A), the 1/8 power had diapedesis of blood cell components within ciliary body vessels (Figure 6B, upper). No changes in pigmented epithelial cells of the ciliary body were observed. Damaged mitochondria and unarranged basal infoldings in nonpigmented epithelial cells with contraction were noted (Figure 6B, below). In the 1/16 power, the capillary vessels in pars plicata were swollen, but there was no diapedesis of blood cell component (Figure 6C, upper). The pigmented epithelial cells were normal, but the nonpigmented epithelial cells had damaged crista in the mitochondria and destroyed basal infoldings accompanied by proliferating tissue (Figure 6C, below). The 1/32 power had normal nonpigmented epithelial cells and only slight atrophy in basal infoldings (Figure 6D).

**Discussion**

Rabbits have an eye axis of approximately 17 mm, limbus sclera approximately 0.5 mm thick, and ciliary body approximately 1.5–2 mm long and 0.3 mm thick [24]. Humans have an eye axis of approximately 24 mm, limbus sclera approximately 0.8 mm thick, and ciliary body approximately 5.5 mm long and 1.2 mm thick. Thus, rabbit and human eyes significantly differ in size. In humans, aqueous humor transits only plasma components to the nonpigmented epithelial side via the fenestrated structure of capillary vessels in the pars plicata derived from the greater arterial circle of the iris, which is composed of the long posterior ciliary artery and the anterior ciliary artery. Aqueous humor is produced by active transport of Na-K ATPase and ultrafiltration determined by static and oncotic pressure and is transported to the posterior chamber [25]. As rabbits produce aqueous humor in the same manner, we used this species in this study because the IOP of humans and rabbits may be decreased by restraining aqueous humor production by cyclophotocoagulation.

Despite the interspecies anatomical differences, effective reduction in IOP was observed in the rabbits treated with the 1/8 and 1/16 power. A diode laser is absorbed into pigmented epithelial cells [9,20], but no changes were observed in the pigmented epithelial cells of rabbits...
treated with the 1/8 and 1/16 power when observed under a microscope. However, the rabbit treated with the 1/8 power exhibited hemorrhage in the ciliary body when observed under a dissecting microscope. Concerning tissue findings, decreased and destroyed ciliary body capillary vessels, damaged mitochondria, and destroyed basal infoldings in nonpigmented epithelial cells were observed. The rabbit treated with the 1/16 power had a slightly swollen ciliary body when observed with the naked eye. Swollen capillary vessels of the ciliary body, damaged crista of mitochondria, and damaged basal infoldings in nonpigmented epithelial cells were observed. The IOP reduction rate and tissue changes conformed. The rabbit treated with the 1/8 power exhibited a large IOP reduction rate but greater tissue invasion. The rabbit treated with the 1/16 power had proliferating tissue associated with repair processes, although the basal infoldings were destroyed. Furthermore, there was no decrease in mitochondrial ATP activity in the nonpigmented epithelial cells.

The mechanisms of MP-TSCPC [9,19,20] are believed to involve the opening of the uveoscleral pathway and aqueous humor dysfunction caused by stimuli from the ciliary body, but detailed mechanisms remain unclear. As one of the mechanisms, our findings suggested that the aqueous humor dysfunction causes an IOP decrease due to the degradation of aqueous humor production caused by the dysfunction of nonpigmented epithelial cells and damaged epithelial basal infoldings in the pars plicata. However, the transport dysfunction of aqueous humor may be reversible because proliferating tissue produced in the process of repairing tissue damage, and sustained ATP activity, which indicates mitochondrial viability.

The characteristics of MP-TSCPC [9] are that all types of glaucoma diseases are adaptive, and MP-TSCPC can be performed repeatedly for glaucoma patients. This differs from conventional CW-TSCPC. In this study, the reversible morphological changes after MP-TSCPC indicated that the MP-TSCPC can be performed repeatedly. Moreover, we emphasized that there is a power setting which is both safe and effective, which is an advantage for clinical applications. As a procedure for humans, the power settings that effectively reduce IOP are preferred because they reduce invasion and complications in glaucoma patients. The rabbit treated with the 1/16 power had less invasion, although IOP reduction was more effective in the rabbit treated with the 1/8 power. Moreover, only slight damage was observed in the stratified squamous epithelial cells in the treated area of the conjunctiva, and there was no adhesion of the conjunctiva or thinning of the sclera. While the present study was conducted on rabbit eyes, we hypothesize that MP-TSCPC may be safely used for human eyes. Glaucoma patients who have a high IOP after trabeculotomy may require trabeculectomy to decrease IOP. However, with a trabeculectomy, there is a risk of intraocular inflammation even though the
procedure can significantly and adequately decrease IOP. Therefore, based on our morphological findings, MP-TSCPC may have clinical applications to reduce IOP in patients with an IOP increase after trabeculotomy and it may be sufficient for patients who choose not to undergo a trabeculectomy.

There were a couple of limitations regarding these experimental procedures. First, the small sample size is problematic in that only one rabbit per each laser power was used. Second, we did not check whether or not the uveoscleral pathway was affected by the treatment. The opening of the uveoscleral pathway should also be verified to confirm other mechanisms of MP-TSCPC.

In conclusion, a mechanism underpinning MP-TSCPC is IOP reduction accompanied by aqueous humor dysfunction induced by dysfunction of nonpigmented epithelial cells and damaged basal infoldings of the pars plicata.

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Statement of Ethics
All protocols were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with the approval of the Animal Experiment Ethics Committee of Kitasato University (approval number: 2018-183).

Disclosure statement
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Author contributions
Design and conduct of the study (HI, KA, KM, SU, NS). Collection, management, analysis, and interpretation of the data (TT, YK, KM). Preparation, review, and approval of the manuscript (TT, HI, KU, KA, KM, YK, NS).
References

Figure Legends

Figure 1. Progress of IOP in rabbits
The rabbit treated with the power setting corresponding to that of humans (1 power) had pre-IOP of 16.7 ± 0.6 mmHg. IOP decreased and reached 3 mmHg (IOP reduction rate of approximately 82%) the following day (1D). The rabbit treated with the 1/8 power setting (1/8 power) had pre-IOP of 17.0 ± 1.0 mmHg, 13.3 ± 2.5 mmHg (22%) the following day (1D), 7.7 ± 0.6 mmHg (55%) after 3 days (3D), 6.0 ± 1.0 mmHg (65%) after 5 days (5D), and 7.0 ± 0.0 mmHg (59%) after 1 week (1W). The rabbit treated with the 1/16 power setting (1/16 power) had pre-IOP of 17.3 ± 0.6 mmHg, 11.3 ± 1.5 mmHg (35%) the following day, 11.3 ± 0.6 mmHg (35%) after 3 days, 8.7 ± 0.6 mmHg (50%) after 5 days, and 10.3 ± 0.6 mmHg (40%) after 1 week. The rabbit treated with the 1/32 power setting (1/32 power) had pre-IOP of 16.7 ± 0.6 mmHg, 15.3 ± 1.1 mmHg (8%) the following day, 16.7 ± 0.6 mmHg (0%) after 3 days, 15.7 ± 0.6 mmHg (5%) after 5 days, and 17.3 ± 0.6 mmHg (4% rise) after 1 week.

Figure 2. Dissecting microscopy
Anterior ocular segments observed with a slit lamp. A Control: sclera, pars plana, pars plicata, and lens from the top. B 1/8 power setting: partial thinning and hemorrhage at the end of pars plicata. C 1/16 power setting: swelling of whole pars plicata. D 1/32 power setting: narrowing of a portion of the pars plicata, but no tissue changes.

Figure 3. Light microscopy of ciliary body
A Control eye: sclera (S) on the left, conjunctiva (C) on the top, Fontana’s space in between the two, ciliary body (CB) on the bottom (scale bar = 100 µm). B 1/8 power setting: enlarged ends of the pars plicata and hemorrhage (black asterisks). Enlarged capillary vessels of ciliary body have disappeared (scale bar = 100 µm). C 1/16 power setting: inflected and folded pars plicata are observed (white asterisks) (scale bar = 100 µm). D 1/32 power setting: inflected pars plicata but with normal appearance (scale bar = 100 µm).

Figure 4. Metallic salt reaction of ciliary body
Evaluation of mitochondrial ATP activity by metallic salt reaction. A Control eye (scale bar = 50 µm). B 1/16 power setting: disturbance associated with destroyed basal infoldings of pars plicata was observed (black arrows), but the ATP activity as the control was observed (scale
bar = 50 µm). C 1/32 power setting: no damage of basal infoldings of pars plicata was observed (scale bar = 50 µm).

**Figure 5. Light microscopy of conjunctiva**
A Control eye (scale bar = 20 µm). B 1/16 power setting: observed damage only in stratified squamous epithelium cells, which match the treated areas (black arrow) (scale bar = 20 µm). C 1/32 power setting: no damage to stratified squamous epithelium cells (scale bar = 20 µm).

**Figure 6. Electron microscopy of ciliary body**
A Control eye: ciliary body (CB) and capillary vessels (C) from the top; pigmented epithelial cells (PE) and nonpigmented epithelial cells (NPE) below (upper) (scale bar = 8 µm). Nucleus (N), mitochondria (m), and basal infoldings (Bi) in an enlarged nonpigmented epithelial cell (below) (scale bar = 2 µm). B 1/8 power setting: diapedesis of blood cell components from capillary vessels of ciliary body (white asterisk) (upper) (scale bar = 10 µm). Damaged mitochondria (white arrows) and unarranged and contracted basal infoldings (black arrow) in an enlarged nonpigmented epithelial cell (below) (scale bar = 4 µm). C 1/16 power setting: no diapedesis of blood cell components in the capillary vessels in pars plicata (upper) (scale bar = 10 µm). Damaged crista (white arrows) in mitochondria and contracted and damaged basal infoldings accompanied by proliferating tissue (black arrow) (below) in an enlarged nonpigmented epithelial cell (scale bar = 2 µm). D 1/32 power setting: almost normal findings (upper) (scale bar = 6 µm). Slight contraction of basal infoldings can be observed in an enlarged nonpigmented epithelial cell but no disturbances in arrangement (below) (scale bar = 2 µm).
Table 1  Treatment conditions

<table>
<thead>
<tr>
<th>Power (mW)</th>
<th>Time (sec)</th>
<th>Duty cycle (%)</th>
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</thead>
<tbody>
<tr>
<td>1 power</td>
<td>2,000</td>
<td>160</td>
</tr>
<tr>
<td>1/8 power</td>
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<tr>
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<td>40</td>
</tr>
<tr>
<td>1/32 power</td>
<td>250</td>
<td>40</td>
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1 power: power setting corresponding to that for glaucoma in humans, 1/8 power: 1/8 power setting of that for humans, 1/16 power: 1/16 power setting of that for humans, 1/32: 1/32 power setting of that for humans.
Figure 1

IOP (mmHg)

Mean ± SD
Figure 5